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Hemophilioid Factors: Acquired Deficiencies in Several Hemorrhagic States. (23030)

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It is rather well established that at least 2 types of congenital "hemophilioid" disease exist, in addition to true hemophilia. In each case, sluggish clotting of blood can be corrected in large measure by addition of either of the other types of blood. Such evidence provides the chief grounds for considering the 3 types as separate entities. It has been postulated by some that these 3 disorders are due to the presence of inhibitors in the blood stream(1). It is more widely held however, that they are due to deficiency of 3 rather ill-defined factors needed for activation of thromboplastin. The 3 factors in question have been referred to as antihemophilic factor (AHF), plasma thromboplastin component (PTC)(2) and plasma thromboplastin antecedent (PTA)(3).

Although the nature of hemophilioid factors is undetermined, study of their activity in coagulation disorders has yielded data which are useful in classification and management of these disturbances. Their levels in acquired hemorrhagic states have never been fully described. In a previous publication it was pointed out that faulty adsorption of vit K leads to lowering of serum level of PTC(4). In the present study, activity of PTA is also shown to be affected under similar conditions. The effects of parenchymatous liver-cell damage on hemophilioid factors will also be discussed.

Methods. Labile factor was measured by Wolf's(5) modifications of Quick and Stefanini's technic(6). *Stable factor* was measured by modification of the method of Owren (7). *Prothrombin* was measured by the 2-stage method of Ware and Seegers(8), modified by substitution of purified labile factor (9) and stable factor(9) in place of dilute bovine serum to stabilize the assay. The *Prothrombin time* test of Quick(10) is now known to measure combined deficiencies of stable factor, labile factor, prothrombin and more rarely of fibrinogen. A dilution technic was used, and all results were expressed as per cent of normal. Methods for quantitative assay of hemophilioid factors have been outlined in principle(4). They were based on the observation of Biggs *et al.* that platelets together with calcium, AHF, PTC and PTA can form a potent thromboplastin(11). In performing the assays we devised a standard procedure whereby a reaction mixture

containing platelets and calcium was supplied with 2 of the 3 hemophilioid factors, each in high titer. The third factor, the one to be assayed, was added as an unknown preparation. The titer of thromboplastin thus produced provided a measure of the hemophilioid factor in question. Plasma and sera with congenital deficiencies of the various hemophilioid factors are used as substrates for these assays. In our laboratory, identification of these deficiencies was made by cross matching plasma or serum with those of our earlier cases or with those from other laboratories. Plasma and serum having a deficiency of PTA were identified by cross testing with plasma obtained from Robert Rosenthal who first defined the deficiency (3).

The preparation of assay reagents, as well as details of the PTC assay, have been described(4). In assay of PTA, as now described, the serum to be assayed is the source of the factor. PTC is supplied by serum from a patient congenitally deficient in PTA but rich in PTC. Adsorbed plasma from a patient rich in AHF but congenitally deficient in PTA is used as a source of AHF. With these modifications, the PTA assay is performed as described already for assay of PTC(4). To assay for AHF, a test plasma, adsorbed with $Al(OH)_3$, is the source of AHF. Normal pooled serum serves as a source of PTC and PTA. $Al(OH)_3$-adsorbed plasma from patient congenitally deficient in AHF is also added; it serves as source of labile factor, the absence of which might influence the assay. The reagents already described(4) are mixed as follows: *Solution A*: 0.1 cc $Al(OH)_3$-adsorbed test plasma (unknown or normal control plasma); 4.4 cc 0.85% sodium chloride; 0.5 cc $Al(OH)_3$-adsorbed plasma congenitally deficient in AHF. *Solution B*: 0.2 cc normal serum diluted 1:10 with 0.85% sodium chloride; 0.2 cc human platelet suspension; 0.2 cc 0.02 M $CaCl_2$. Next, 0.2 cc of Sol. A are incubated at 37°C with 0.6 cc of Sol. B. Remainder of assay is performed as described for assay of PTC(4).

Results. Activities of hemophilioid factors were compared with activities of other clotting factors in a variety of acquired hemorrhagic states:

Hepatocellular disease: Blood specimens were collected from 20 patients having a variety of hepatocellular diseases, and from 30 normal controls. Various coagulation factors were measured, with special emphasis on PTA, PTC and AHF. In addition, hepatic status was evaluated on clinical grounds and by performing routine tests of hepatic function. Patients with liver disease were divided into 2 groups, acute and chronic (Table I). The former included infectious hepatitis and homologous serum hepatitis; the latter included portal cirrhosis, Wilson's disease, Gaucher's disease and carcinoma metastatic to the liver. In acute disorders, levels of PTA activity ranged from 21% of normal in patient with severe homologous serum hepatitis, to 43% of normal in patient recovering from infectious hepatitis. In chronic disorders, levels of PTA ranged from 16% in patient who died shortly thereafter with acute yellow atrophy, to 95% of normal in patient with mild portal cirrhosis. PTC values in these patients had a similar range. In both acute and chronic groups, data for serum proteins, bilirubin and cephalin flocculation also varied considerably but these data corresponded roughly with data for the clotting factors. None of these tests differentiated clearly between acute and chronic disorders. Blood levels of PTA and PTC seemed often to provide a more sensitive index of dysfunction than did levels of prothrombin, stable factor or labile factor. This too can be concluded from recent data published on PTC by Cowling (12).

Vit K deficiency: The influence of vit K on prothrombin has been known for many years and its influence on stable factor and PTC has been investigated previously(13,4). Influence of the vitamin on PTA has never been reported. Several patients with inability to adsorb vit K were therefore studied. Diagnoses included non-tropical sprue, obstructive jaundice and fibrocystic disease. Representative data, obtained on patient with obstructive jaundice are shown in Table II.

TABLE I. Levels of Activity of Plasma Thromboplastin Component (PTC), Plasma Thromboplastin Antecedent (PTA) and Other Clotting Factors in Both Acute and Chronic Hepatic Disorders.

	Proth. time											
	1 stage	2 stage	Lab. fac.	Stab. fac.	PTC	PTA	AHF	A/G ratio, g %	Bilirubin total, mg %	Alk. phos., B.U.	Ceph. floc.	Clinical status
	(In % of normal)											
Normal												
Mean avg	100	100	100	100	100	100	100	4.6/2.0	<.8	1-4	0	
Stand. dev. of mean					± 6.2	± 7.4	± 5.9					
Acute hepatocellular disease												
Infectious hepatitis	77	89	85	56	54	43	100	3.8/3.0	4.0	6.7	3	Recovering
Idem	70	100	81	60	68	40	95	4.0/3.3	3.0	7.3	4	"
Serum hepatitis	35	64	80	29	22	21	100	3.0/2.9	26.0	6.3	3	Severe
Idem	75	100	75	67	60	33	95	3.7/2.5	6.3	7.1	3	Recovering
Serum hepatitis and	80	91	90	93	25	37	7	3.3/3.6	16.5	7.4	3	"
hemophilia recovered from hepatitis	100	100	100	100	100	90	5					Recovered
Chronic hepatocellular disease												
Portal cirrhosis	98	87	100	72	87	95	100	3.1/3.9	.7	8.3	4	Mild
Idem	37	43	36	26	28	35	89	2.5/2.9	6.2	3.9	4	Severe
"	37	40	40	34	40	42	97	2.2/3.4	1.4	4.6	4	"
"	74	53	72	60	50	49	100	3.3/3.5	.5	2.7	0	Advanced
"	54	35	28	27	18	21	100	3.2/4.3	4.0		4	"
"	66	59	97	40	66	31	100	4.2/2.6	.8	2.8	4	Compensated
"	69	89	70	60	71	57	95	3.2/1.5	.4	5.8	0	"
"	55	53	43	53	75	32	100	3.6/3.1	1.2	2.7	4	"
"	79	84	67	57	42	46	100	4.9/1.0	.5		2	"
"	45	65	45	22	75	49	100	2.5/2.7	2.0	1.6	4	Severe
" & acute atrophy	9	38	18	5	6	16	80				3	Died in 8 hr
Wilson's disease	87	85	63	39	45	38	100	4.4/1.7		5.4	0	Advanced
Gaucher's disease	64	44	50	33	66	21	100	4.1/2.1	1.5	5.7	0	"
Carcinoma, metastatic to liver	71	100	108	34	53	30	86	4.8/2.8	.9	20.3		Died in 2 wk

TABLE II. Effect of Vit. K Replacement on Levels of PTA, PTC and Other Clotting Factors in Several Patients Unable to Adsorb Fats.

	Proth. time 1 stage	Proth. time 2 stage	Labile factor	Stable factor	PTA	PTC	AHF
	In % of normal						
Obstructive jaundice							
Before treatment	22	66	91	15	40	23	96
26 hr after Hykinone (10 mg)	86	100	89	38	100	75	94
Fibrocystic disease							
Before treatment	11	34	82	9	7	11	100
24 hr after Hykinone (15 mg)	82	79	82	86	73	78	100
Sprue, non-tropical							
Before treatment	5		90	7	9	7	95
28 hr after Vit. K_1 (75 mg)	81		92	79	68	86	95

A deficiency of vit K in this patient was accompanied by depression in levels of prothrombin, stable factor, PTC and PTA. Ability of vit K to correct these deficiencies was tested by intramuscular administration of 10 mg of menadione sodium bisulfite (Hykinone). Twenty-six hours later, all aforementioned clotting factors had either increased by 100% or had returned to normal levels. PTA activity increased from original level of 40% before treatment, to 100% of normal after treatment. Other patients with inability to adsorb vit K showed a similar response when the vitamin was replaced (Table II).

Effect of anti-vit K. Deficiencies of prothrombin(14), stable factor(15) and PTC (4) have been reported. A fall in PTA has not previously been reported. A number of patients were studied while receiving 3-3′-methylenebis (4-hydroxy coumarin) (Dicumarol). Two representative patients, suffering with thrombophlebitis, were treated. Assay values of coagulation factors are shown in Table III. Depressions of prothrombin, stable factor, PTC and PTA accompanied treatment with this compound. Upon recovery from thrombophlebitis, the ability of vit K to correct these deficiencies was tested by intramuscular administration of vit K and Hykinone. Within 24 hours, factors previously depressed had risen almost to normal levels. In one case, PTA activity increased from original level of 22% before treatment to 89% of normal after treatment.

Discussion. Disturbances in blood coagulation associated with hepatic disease are not simple ones. Significant deficiencies of a number of circulating coagulation proteins exist. These deficiencies may be related both to destruction of hepatic cells and to hypovitaminosis K of biliary obstruction. Blood levels of prothrombin, stable factor, labile factor, PTC, fibrinogen and PTA are reduced in disease processes that are predominantly hepatocellular. Levels of prothrombin, stable factor, PTC and PTA are also dependent upon an adequate supply of vit. K. In any

TABLE III. Effect of Vit. K_1 and Hykinone on Levels of Clotting Factors Altered by Administration of Dicumarol.

	Proth. time 1 stage	Proth. time 2 stage	Labile factor	Stable factor	PTA	PTC	AHF
	In % of normal						
Patient A							
Before Dicumarol	100	100	97	100	98	100	95
After 6 days of Dicumarol	26	68	97	17	22	13	95
20 hr after Hykinone (10 mg)	70	89	94	60	89	85	95
Patient B							
Before Dicumarol	100	100	100	100	96	100	100
After 9 days of Dicumarol	11	21	100	13	24	29	100
22 hr after vit. K_1 (40 mg)	48	42	100	51	58	61	100

hepatic disorder, the relative importance of hepatocellular damage and vit. K deficiency varies. Summation of the two frequently attains clinical importance. If the hemorrhagic crisis is predominantly due to hepatic cell destruction, vit K will be ineffective, and deficient factors must often be replaced by transfusion of blood or plasma. Fortunately, stored blood preparations commonly available retain high levels of PTA, PTC, prothrombin, stable factor and fibrinogen. However, their titers of labile factor are often low. Therefore, when significant deficiencies of labile factor exist, hemostasis may be restored only by transfusion of fresh blood or plasma. Thus it is that analysis of specific deficiencies may be important.

Dependence of several coagulation factors upon hepatocellular function is not surprising, since a wide variety of blood proteins are similarly dependent. However, several of these factors have other properties which suggest that the relationship between them may be a close one. Prothrombin, stable factor, PTC and PTA have physical properties in common (*e.g.* precipitation, adsorption, stability). In addition, these 4 clotting factors must share at least one common metabolic pathway since all 4 are dependent upon vit K. Other blood proteins have not been shown to be dependent upon this vitamin.

Summary. Deficiencies of hemophilioid factors are not necessarily genetic in origin. Serum activities of plasma thromboplastin antecedent (PTA) and of plasma thromboplastin component (PTC) were depressed in 20 patients with a variety of hepatocellular disorders. PTA and PTC activities were also reduced in several patients who developed a deficiency of vit K subsequent to inability to adsorb fats. PTA and PTC were depressed in a number of patients following administration of 4-hydroxy coumarin compound. In these latter 2 groups, PTA and PTC activities rapidly increased toward normal following administration of vit K analogue. Significant deficiency of antihemophilic factor (AHF) was not found in any of the above conditions.

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Isolation and Identification of a New Ketone Body in Normal Human Urine.* (23031)

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Acetoacetic acid, its reduction product, β-hydroxybutyric acid, and its decarboxylation product, acetone, have long been considered the ketone bodies of blood and urine. In the course of a study involving quantitative determination of dihydroxyacetone in urine, we observed interference by a neutral ketone other than acetone on the colorimetric assay of the triose. This report describes the isolation and identification, and discusses the possible origin of a new ketone body, methyl ethyl ketone, in human urine.

Materials and methods. Ten liters of pooled urine from 5 apparently healthy adults collected under toluene in sterile containers were acidified with 800 g of metaphosphoric acid, and the small amounts of protein and other colored material were filtered with the aid of Celite. To the filtrate was added 42 g of recrystallized 2,4-dinitrophenylhydrazine hydrochloride prepared as a saturated solution, in warm 2 *N* HCl. After 48 hours' standing at 25°, the dark orange precipitate was collected on a sintered glass funnel with a layer of Celite over the filtering surface. The contents of the funnel were washed twice with 250 ml of water and subjected to suction for removal of water. Precipitate and Celite were extracted with ethyl acetate repeatedly until no more colored material came off the Celite. The combined extracts measured about 2 l and were washed with 2 *N* HCl until the washings became nearly colorless, to remove unreacted 2,4-dinitrophenylhydrazine and other acid-soluble impurities. Then the ethyl acetate solution was washed repeatedly with 5% $NaHCO_3$ solution until washings became nearly colorless to remove hydrazones of keto acids. Finally the organic layer was washed with water and the solvent removed by stream of air overnight. The black syrupy residue was taken up in 100 ml of hot 95% alcohol and the solution was treated with 0.5 g of Norite charcoal, then filtered. The filtrate yielded a gummy precipitate on cooling. This process of purification was repeated 6 more times with minimum amounts of alcohol so that crystalline material was finally obtained. After 3 recrystallizations of the product from 95% alcohol and two recrystallizations from 50% alcohol, the pure material in the form of long plates had a melting point of 111-112° (corr.). Approximately 50 mg of 2,4-dinitrophenylhydrazone of the unknown neutral ketone was isolated from 10 l of urine.

Results. For identification of the unknown ketone the recorded melting points of 2,4-dinitrophenylhydrazones of known ketones were surveyed; methyl ethyl ketone had a melting point of 111°(1). The 2,4-dinitrophenylhydrazone of commercial methyl ethyl ketone (Eastman Kodak Co.) was prepared and the identity of the derivative of the unknown ketone with that of methyl ethyl ketone was established by the following observations: a) the mixed melting point showed no depression, b) chromatogram on Whatman No. 1 filter paper developed with methanol-saturated heptane(2) showed identical R_f of 0.64 (3), c) absorption spectra in 0.25 *N* NaOH (in 70% alcohol) were identical, showing the same maxima at 434 mμ and 535 mμ.

Discussion.† The most likely precursor of methyl ethyl ketone is α-methylacetoacetic acid which has been suggested as an intermediate of isoleucine catabolism(4,5). The steps in pathway from isoleucine to methyl ethyl ketone may be postulated as follows: oxidative deamination, decarboxylation, β-oxidation of long carbon chain(6), and finally decarboxylation. Since this ketone was found in the steam distillate of mulberry leaves(7),

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one must not overlook the possible dietary origin of methyl ethyl ketone in normal human urine.

Summary. Methyl ethyl ketone has been isolated from normal human urine and identified. Its possible origin was briefly discussed.

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Vit. B_{12} Levels in Serum and Urine Following Parenteral Administration of Crystalline Vit. B_{12} or Liver Extract.* (23032)

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Sokoloff, Sanneman, and Beard(1) on the basis of urinary excretion studies have suggested that there may be better retention of vit. B_{12} activity in tissues following parenteral administration of purified liver extract than following an equal amount of crystalline vit. B_{12}. It has been postulated(2) that vit. B_{12} must be bound to serum proteins to prevent its excretion and therefore facilitate its ultimate retention in tissues. This paper compares serum and urine vit. B_{12} levels of 2 groups of normal subjects, one group given crystalline vit. B_{12}, the other given an equivalent amount of vit. B_{12} in the form of purified liver extract.

Materials and methods. Subjects for this study were students and staff of Tulane University School of Medicine, and selected patients from Charity Hospital. One group of these subjects was given 50 μg of crystalline vit. B_{12}† in 1.0 ml of solution. The other group was given an amount of purified liver extract‡ containing 50 μg of vit. B_{12}, in 3.3 ml of solution. Both vit. B_{12} solution and liver extract were assayed prior to use by microbiological technic using *L. leichmannii* (ATCC-4797). In both groups, blood samples were drawn before injection, and at 1, 4, 8 and 24-hour intervals following injection. Urine was collected for 24 hours following injection. Diet was not controlled, as normal diets were not found to affect urinary excretion or serum levels of vit. B_{12}(3,4). Serum was separated from blood samples and stored at –20°C until assayed. From each sample of serum collected, an aliquot was treated with acid-washed charcoal, 75 mg/ml to adsorb free vit. B_{12}(5). These aliquots, along with untreated aliquots, were then assayed for vit. B_{12}§(6). Aliquots of 24-hour urine samples were stored at –20°C until assayed (3,6). Serum vit. B_{12} levels are expressed as mμg/ml and urinary excretion values are reported as μg/24 hours. To approximate the average amount of bound vit. B_{12} in serum over 24 hours and to relate this value to the average total vit. B_{12}, bound and total levels of the vitamin, of each subject, were plotted on coordinate graph paper for 0, 1, 4, 8, and 24 hours following the dose. The points were

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† "Cobione" was supplied by Merck Co., Rahway, N. J.

‡ Liver extract was supplied by Lederle Division of Am. Cyanamid Corp., Pearl River, N. Y.

§ "Vit. B_{12}" and "Vit. B_{12} activity" will be used synonymously.

TABLE I. Vit. B_{12} Values (mμg/ml) in Serum following Administration of 50 μg Crystalline Vit. B_{12} or Its Equivalent as Liver Extract.

Time of sampling, hr	Form of vitamin administered: Crystalline (8) Total	Crystalline (8) Bound	Crystalline (8) % bound	Liver extract (8) Total	Liver extract (8) Bound	Liver extract (8) % bound
Fasting	.27 ± .04*	.19 ± .04	70.4	.24 ± .03	.21 ± .03	87.5
1	1.74 ± .15	.75 ± .06	43.1	.96 ± .11	.70 ± .09	72.9
4	.94 ± .05	.69 ± .04	73.4	1.10 ± .08	.77 ± .03	70.0
24	.52 ± .04	.41 ± .04	78.8	.47 ± .04	.39 ± .03	82.9
Avg % bound, calculated†/24 hr period			64.9 ± 3.4			81.5 ± 2.5

* Stand. error of mean. † Calculations described under methods.
No. in parenthesis is No. of subjects in group.

connected to form rough time-course curves, and the area under the curves determined. Average bound vitamin is expressed as % of total vit. B_{12}.

Results. Values for total vit. B_{12} in serum, drawn post absorptively, averaged 0.26 mμg (Table I) which agrees with values previously reported by this method(4,5,7). Bound vit. B_{12} values of all subjects in post absorptive state averaged 0.20 mμg, which is about 77% of total vit. in serum, and is in agreement with data obtained previously(5,8,9). Serum vit. B_{12} levels, both total and bound, determined 24 hours following injection of the vitamin are higher in both groups than pre-injection fasting levels (Table I).

Average maximum concentrations of total vit. B_{12} and bound vit. B_{12} were 1.8 and 0.8 mμg respectively in the group given crystalline vitamin. In contrast, average maximum concentration of total vit. B_{12} was lower in the group receiving liver extract, 1.2 compared to 1.8 mμg. However, average maximum concentration of bound vitamin was the same in both groups.

The time required for maximum vit. B_{12} level in serum to be reached was different in the 2 groups. In the group given crystalline vitamin, 7 of 8 subjects reached maximum values in one hour, however, 6 of 8 subjects given liver extract required 4 hours. These observations are reflected in data shown in Table I. One hour after injection of vit. B_{12}, average level in serum was greater in the group receiving crystalline vitamin than in the group receiving liver extract, although no difference was observed in serum levels taken at 4 hours.

Average proportion (%) of bound vitamin to total vitamin was calculated over the 24 hour period as described above. Data shown in Table I demonstrate that a greater % of vit. B_{12} was bound following administration of liver extract than was observed following injection of crystalline vitamin, 81.5 ± 2.5% and 64.9 ± 3.4% ($p<0.01$). These data suggest that a greater proportion of vitamin is free, following administration of crystalline vit. B_{12}, and therefore it would be anticipated that a greater amount of vit. B_{12} would be excreted in urine following administration of crystalline vit. B_{12}, rather than liver extract. Urinary excretion of the vitamin in the group receiving crystalline vit. B_{12} (16 subjects) averaged 9.0 ± 0.75 μg as compared to 6.0 ± 1.1 μg for 8 subjects that received liver extract. The groups are significantly different at the 2% level.

Although crystalline vit. B_{12} is completely adsorbed on charcoal and completely dialyzable against 100 volumes of saline; vit. B_{12} in liver extract, although completely adsorbed on charcoal, was only 67% dialyzable under these conditions. It seemed probable that the differences in urinary excretion resulted from unavailability of the vitamin to the blood and hence to the kidney, when administered as liver extract.

Discussion. Ability of human serum to bind vit. B_{12}, *in vitro* and *in vivo*, has been studied by several investigators(5,7,8,9). Mollin and Ross(2) studied this phenomenon in normal subjects, and showed that human serum has a limited ability to combine with vit. B_{12}, with maximum serum bound vit. B_{12} levels of 0.8 to 1.4. It is possible that bind-

ing of vit. B_{12} with serum may be necessary to prevent its urinary excretion, and that the binding mechanism is capable of handling a limited amount of absorbed vitamin. Urinary excretion of vit. B_{12} may be proportional to the level of free vitamin in serum(2), although this point has not been confirmed in this laboratory.

Upon examination, the data demonstrate that total concentration of vit. B_{12} in serum is not the only factor influencing maximum concentration of bound vit. B_{12}. This is suggested by the observation that maximum concentration of serum bound vitamin is not different in the 2 groups one hour after administration of the vitamin while maximum concentration of total vitamin is different. However, concentration of total vit. B_{12} does influence to some degree the concentration of bound vitamin, inasmuch as the highest bound vitamin levels occurred at 1 hour in the group receiving crystalline vitamin and at 4 hours in the other group.

The higher proportion (%) of bound vitamin in serum of the group receiving liver extract was probably due to slower absorption from site of injection, inasmuch as total concentration of vit. B_{12} in serum does not seem to be directly related to the binding maximum. This slower absorption, perhaps influenced by the non-dialyzable fraction of liver extract, resulted in less vitamin present in free or uncombined form. However, absorption was rapid enough to saturate the binding mechanism. This slower absorption continued over a longer period of time, and apparently resulted in less excretion of the vitamin by the kidney.

Summary. Liver extract and crystalline vit. B_{12} were administered in equivalent amounts to 2 groups of normal subjects, and total serum vit. B_{12} levels, bound vit. B_{12} levels, and urinary excretion of vit. B_{12} compared in the 2 groups. Concentration of free vit. B_{12} was higher in the group receiving crystalline vitamin. Over the 24-hour period, percentage of bound vitamin serum was greater in the group receiving liver extract. Urinary excretion of vit. B_{12} was significantly lower in the group receiving liver extract.

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Studies on Conversion of Phenylalanine to Tyrosine in Phenylpyruvic Oligophrenia.* (23033)

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Recent studies(1,2) indicate that patients with phenylpyruvic oligophrenia have markedly reduced ability to convert phenylalanine to tyrosine. The enzyme system that catalyzes this conversion was demonstrated in mammalian liver preparations by Udenfriend and Cooper(3), and subsequently Jervis(4) reported that this enzyme activity was lacking in livers of 2 patients with phenylpyruvic oligophrenia, whose tissues became available for study at autopsy. No studies on freshly obtained liver have apparently been reported; the lability of this enzyme system suggests the desirability of such study. Mitoma(5) recently made the interesting observation that rat phenylalanine oxidizing system can be separated into two protein fractions; one of these (Fraction I) was relatively labile and was found solely in liver, while the other (Fraction II) was more stable and found also in certain other tissues. We have been fortunate in obtaining a biopsy specimen of liver from a patient† with phenylpyruvic oligophrenia, and have carried out studies on the ability of this preparation to catalyze conversion of phenylalanine to tyrosine in the presence and absence of the 2 purified rat liver fractions.

Methods. Liver samples were placed in cracked ice within a few minutes of excision, and homogenized within 10 minutes with 2 parts of ice-cold 0.15 M KCl. Rat Fractions I and II were obtained as described(5). The reaction mixtures consisted of diphosphopyridine nucleotide (0.06 μM), nicotinamide (0.5 μM), ethanol (2 μM), L-phenylalanine-3-C^{14} (0.4 μM; 56,000 cpm), and 0.1 ml of homogenate (or 0.05 ml of rat fraction); final volume was 0.20 ml. The mixtures were incubated 30 minutes with shaking at 37°. After incubation, one-third of the reaction mixture was transferred to a paper chromatographic strip (30 x 2 cm; Whatman No. 3), developed in a solvent consisting of n-butanol, acetic acid, and water (4:1:1). After ascending chromatography for 12 hours, the strips were cut into one cm sections and counted in a flow-gas tube. Under these conditions, the R_F values for tyrosine and phenylalanine were, respectively, 0.38 and 0.66. The % conversion was calculated from the cpm in the tyrosine area and the total cpm on each strip. No significant radioactive material was found in other than the phenylalanine and tyrosine areas. The values given in Table I represent the averages of 2 closely agreeing duplicate experiments. Homogenates used in these experiments were frozen immediately after use; repetition of experiments 3 weeks later with freshly-prepared purified rat fractions gave very similar results.

Results. As indicated in Table I, the homogenate prepared from the liver of the phenylketonuric patient did not catalyze detectable conversion of phenylalanine to tyrosine. Under experimental conditions employed, a conversion of 0.5% could have been

TABLE I.

Tissue preparation	% conversion
Phenylketonuric liver homogenate	0
Idem + rat Fraction I	76.4
" + " " II	12.3
Rat Fraction I	9.2
" " II	3.3
" " I + rat Fraction II	24.0
Rat liver homogenate	21.9
Normal human liver homogenate	7.8
Idem	10.2
"	14.1

* This research was supported in part by grants from N.I.H., Public Health Service, Bethesda, Md. The authors acknowledge the skillful assistance of Miss Rita Indresano.

† The patient was a 4-year-old white male exhibiting characteristic biochemical and clinical signs of phenylpyruvic oligophrenia; the authors are indebted to Dr. Marshall Kreidberg of Boston Floating Hospital for making this patient available for study and for his cooperation and interest.

detected. This result contrasts with those obtained with homogenates prepared from several normal human livers‡ and livers of rats. Rat liver Fractions I and II, in confirmation of Mitoma, catalyzed much lower conversion when studied alone than when assayed together; values for rat Fractions I and II together were about twice the sum of the individual values for the 2 fractions. When rat Fraction I and the phenylketonuric liver preparation were incubated together, a striking increase in conversion of phenylalanine to tyrosine was observed; much less activity was noted when the phenylketonuric preparation and rat Fraction II were incubated together. These observations are consistent with the conclusion that the liver of the phenylketonuric patient did not catalyze conversion of phenylalanine to tyrosine. They also suggest that the more labile protein fraction, apparently present only in liver is lacking in phenylketonuric liver. The smaller activation observed with rat Fraction II may probably be ascribed to incomplete separation of the fractions; it is evident that neither of the rat fractions is completely free of the other.

The striking activation observed with phenylketonuric liver homogenate and rat Fraction I together suggests not only that a component corresponding to rat Fraction I is lacking, but that considerable quantities of the component corresponding to rat Fraction II are present in the phenylketonuric liver. Similar studies carried out with normal human liver homogenates and homogenates of rat liver indicated that rat Fraction I produced activation, although less than that observed with the phenylketonuric liver preparation. The latter experiments are consistent with the belief that in crude homogenates the component corresponding to Fraction II is present in large amounts, and also with the observed lability(5,6) of Fraction I. However, other explanations are not excluded, and it is evident that final conclusions cannot be made until the detailed mechanism of this enzymatic reaction is elucidated. The present findings and those reported in the accompanying paper(6) suggest that the defect in phenylpyruvic oligophrenia is related to the absence of a specific hepatic factor rather than one which is present in a number of tissues including brain.

Summary. A homogenate of a biopsy specimen of liver obtained from a patient with phenylpyruvic oligophrenia did not catalyze detectable conversion of phenylalanine to tyrosine, under conditions whereby liver homogenates obtained from normal individuals were active in catalyzing such conversion. Addition of a fraction prepared from rat liver (which exhibited relatively low activity alone) to the inactive phenylketonuric liver homogenate resulted in striking activation of the conversion of phenylalanine to tyrosine.

‡ The authors wish to thank Drs. A. Callow, H. Miller, H. Rheinlander, H. Sugarman, and R. P. Johnson of the New England Center Hospital, and Professor C. G. Child of Boston City Hospital for providing liver specimens used in these studies. The "normal" specimens were obtained from 3 patients with gall bladder disease.

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On the Nature of Enzymatic Defect in Phenylpyruvic Oligophrenia. (23034)

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(Introduced by A. Meister)

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In a previous communication(1) from this laboratory, it was reported that phenylalanine hydroxylase consists of 2 protein fractions; one of which (Fraction I) is present only in the liver and the other (Fraction II) in almost all tissues, including brain.

Since patients with phenylpyruvic oligophrenia are essentially devoid of phenylalanine hydroxylase activity(2,3,4), the question arises as to which, if not both, of the 2 fractions may be absent in this disorder. In the present communication, data are presented to show that Fraction II is present in phenylketonuric patients and some of the implications of these findings are discussed.

Methods. Liver samples* from normals and phenylketonuric patients were obtained at autopsy, 4 hours after death, and kept in frozen state until use. Four grams of liver were thawed, minced and homogenized in 3 volumes of isotonic KCl. Fractions I and II from both human liver and rat liver were prepared, as described previously(1); the terms, purified enzyme fraction and crude enzyme fraction, used in the Tables, refer to terms used in that paper. The incubation conditions and the method for enzyme assay were also the same as used before(5).

Results. As shown in Table I, conversion of phenylalanine to tyrosine was catalyzed upon recombination of rat Fraction I with rat Fraction II. In these studies combination of Fractions I and II of human liver, both normal and phenylketonuric, failed to catalyze this reaction. This was apparently due to failure of Fraction I, in normal liver, to survive during the several hours of autolysis between death and autopsy, since fresh human liver can catalyze this reaction(4,5). However, stable Fraction II survived under these conditions. Thus, when rat Fraction I was incubated with Fraction II from the phenylketonuric liver, formation of tyrosine took place. Furthermore, as shown in Table II equal amounts of Fraction II, from both phenylketonuric and normal livers, catalyzed formation of tyrosine to the same degree, when they were combined with rat Fraction I.

It is apparent from this that Fraction II is present in phenylketonuric livers in normal amounts indicating that in these patients the block in tyrosine formation is more likely associated with absence of Fraction I. Unfortunately, the lability of Fraction I in the normal autopsy samples made it impossible to show this directly. Similar studies on fresh biopsy material will be required to establish

TABLE I. Presence of Fraction II in Phenylketonuric Liver.

Enzyme preparations*	μmole of tyrosine formed
RI	.0 †
RII	.0
RI + RII	.44
PI + PII	.0
PI + RII	.0
RI + PII	.40

* Purified enzyme fractions were used in these experiments. RI—Rat liver Fraction I, 13.5 mg/beaker. RII—Rat liver Fraction II, 6.9 mg/beaker. PI—Phenylketonuric liver Fraction I, 17 mg/beaker. PII—Phenylketonuric liver Fraction II, 15.8 mg/beaker.

† Limit of detection of enzymatically formed tyrosine by the colorimetric method used was 0.05 μmole of tyrosine.

All beakers contained 2 μmoles of L-phenylalanine, 0.5 μmole of DPN, 5 μmoles of nicotinamide, 400 μmoles of glucose, 325 units of glucose dehydrogenase and enzyme fractions in final vol of 2.2 ml. Incubation was carried out 15 min. at 37° in a Dubnoff metabolic shaking incubator under air. The data presented above represent typical values obtained after several experimental runs.

* The patient was a 2-year-old white female exhibiting characteristic chemical and clinical symptoms of phenylpyruvic oligophrenia. The normal liver, data for which are shown in Table II, was also obtained from a 2-year-old, white female. Another sample of liver was obtained from a 6-month-old, white male. The authors are indebted to Sister Fernandes of the Pathology Department of Georgetown Hospital for providing us with the autopsy samples.

TABLE II. Comparison of Fraction II Activity in Normal and Phenylketonuric Livers.

Enzyme preparations*	μmole of tyrosine formed
RI	.15
RI + RII	.98
RI + NII	.82
RI + PII	.81
RI + P**II	.15

* Crude enzyme fractions were used. RI—Rat Fraction I, 26.6 mg/beaker. RII—Rat Fraction II, 10.8 mg/beaker. NII—Normal human Fraction II, 15 mg/beaker. PII—Phenylketonuric Fraction II, 15 mg/beaker. P**II—Phenylketonuric Fraction II, heated 5 min. in boiling water bath, 15 mg/beaker.

Incubation conditions were as described under Table I. Similar results were obtained with NII from a second control patient.

this point. Such studies are reported by Wallace *et al.*, in the preceding paper(4). In that report as well as in this one, experimental findings on tissues from only one phenylketonuric patient are given. It would obviously be desirable to have these findings corroborated on more patients but liver samples from phenylketonuric patients are not readily obtainable.

Since the conversion of phenylalanine to tyrosine takes place only in the liver and Fraction I is found exclusively in this organ, the conclusion that Fraction I is the enzyme primarily concerned with the hydroxylation reaction seems justifiable. Fraction II which is found in many tissues, may then be looked upon as an enzyme concerned with an auxiliary reaction, the nature of which is still not understood. Had Fraction II been missing one might have inferred a disturbance in metabolism in many tissues, including brain. Since, as is suggested by these studies, Fraction I is absent, the primary defect in phenylketonuria must be one of liver function. The chemical anomalies manifested in these patients most probably result from failure to hydroxylate phenylalanine, resulting in an excess of phenylalanine in tissues and an overproduction of other intermediary metabolites of phenylalanine catabolism. Suggestions that the mental deficiency is caused by an overproduction of one of these metabolites or by excess phenylalanine itself(6,7) are supported by these enzymatic findings.

Summary. Evidence is presented to show the presence of the stable Fraction II of phenylalanine hydroxylase in the liver of a patient with phenylpyruvic oligophrenia. The biochemical significance of this finding is discussed.

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Resistance of Dissociants of *Gaffkya tetragena* to Penicillin and Streptomycin.* (23035)

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The ability of bacteria to become resistant to antimicrobials presumably rests on a genetic basis. Current views on the matter are (a) a few originally resistant bacteria exist as variants in any bacterial population and (b) dissociation toward increased resistance is induced by exposure to antimicrobials. In both circumstances the parent population dies and the resistant forms live to perpetuate growth in the presence of the drug. A third view (c) assumes that a gradual adaptive change occurs among bacteria whose progeny are more resistant than the parent strains.

A convenient way to test these views is provided by *Gaffkya tetragena* whose numerous dissociants or variants are distinguishable by their colonial color and texture. In previous studies, a strain derived from a patient grew in its usual S-form white colonies on agar. After many months of observation, its variant S-yellow, S-brown, S-pink and S-pink-yellow types, their respective M and R phases and a colorless translucent form were isolated. Reversions between types occurred and immunologic relationships were demonstrated. Variation apparently developed by chance and could not be forced in any desired direction(1,2).

Methods. In preparation for this study, the same strain preserved for 20 years as *6007* in the American Type Culture Collection, was restudied. By applying methods previously used(1) only the mucoid-white and mucoid-pink colony type appeared after 6 months of observation. Strain *10875* A.T.C.C. then was obtained and in the course of aging for 3 to 6 months at room temperature in broth and on solid agar, the S-yellow, S-brown, and a translucent form were isolated. For unknown reasons, variant colonies appeared among the S-white colonies and not as daughter colonies nor in sectors as previously observed. Small colonies often appeared as usual among the usual large ones of all 6 forms. Subcultures of the large ones reproduced their kind; those of the small ones produced both large and small ones.

Both stock strains seldom grew in concentrations greater than .002 unit/ml penicillin, and of 0.3 unit/ml of streptomycin in broth. Growth in slightly higher concentrations or inhibition of growth by lower concentrations occurred at times, probably due to variations in density of the inoculum, age of the culture seeded, slight differences in dilution in media, in temperature and other variables.

Serial subcultures were made at 24 or 48 hour intervals in broth containing subsuppressive amounts (.001 unit/ml) of penicillin and of (.12 unit/ml) streptomycin. Because of poor growth in broth, passages then were made on agar-plates containing the same amounts respectively. The 6 types were seeded separately on sectors and transferred daily in heavy inoculums from plate to plate. Plates were incubated at 37°C for 12 hours and at room temperature for 3 to 6 days and examined for evidence of visible variation.

Results. Penicillin. During 10 transfers of both stock strains on penicillin-agar, no dissociation was detected. Cocci grown in penicillin-broth or on penicillin-agar often were much larger than those in plain media. There also was no detectable dissociation or reversion, except of one type, among the variants separately transferred 10 times on penicillin-agar nor any evidence of increased resistance. Frequent appearance of a few white colonies among the brown ones indicated a reversion. In several resistance tests of the tenth passage cultures, growth of the white, brown and translucent colonies was suppressed by concentrations higher than .001 unit/ml and of the yellow and mucoid-white colonies, by .002 unit/ml which was identical with the toler-

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TABLE I. Increase of Resistance of Variants of *G. tetragena* to Streptomycin after Growth on Streptomycin-Agar.

		Streptomycin, U/ml							
		10	5	2.5	1.2	.6	.3	.15	Control
Stock strain variants	W		—	—	—	—	±	+	3+
	Y		—	—	—	—	±	+	3+
	B		—	—	—	—	±	+	3+
	M-W		—	—	—	—	±	+	3+
	M-P		—	—	—	—	—	+	3+
	T		—	—	—	—	±	+	3+
After 9 × on streptomycin	W	—	—	—	—	+	+		
	Y	—	—	±	±	+	+		
	B	—	—	—	—	±	+		
	M-W	—	±	+	+	+	+		
	M-P	±	+	+	+	+	2+		
	T	—	—	—	—	+	+		
After 18 × on streptomycin	W	—	—	+	+	2+			
	Y	+	+	+	2+	3+			
	B	—	—	—	±	+			
	M-W	—	+	2+	3+	3+			
	M-P	±	+	+	3+	3+			

3+ to + indicates density of growth on agar plates after 12 hr at 37°C and 5 days at room temp. ± indicates growth of a few colonies only.

ance of the unpassaged cultures. In other words, resistance did not increase.

Streptomycin. In similar serial passages of the stock strains and of their separate variant forms, now including the mucoid-pink type, on agar containing .12 then .25 unit/ml, no variation or reversion was detected, except for the usual appearance of a few white colonies among the brown. A test after the ninth transfer indicated increased resistance of all 6 types as shown in the table. The translucent form was least resistant and died out later; the S-yellow, mucoid-white and mucoid-pink types acquired the highest resistance. In previous studies the S-yellow type was more resistant than the others to adversity(2).

Transfers were continued for 9 more passages on agar containing 0.5 unit/ml. Cultures from the eighteenth passage revealed further increase in resistance of all forms except the brown, and especially of the yellow, mucoid-white and mucoid-pink. Small white, yellow, brown, mucoid-white and mucoid-pink colonies continually appeared among the respective usual large ones, and their tolerance, surprisingly, was slightly greater by one-step higher concentration. In general, growth on agar containing subsuppressive amounts of streptomycin increased the resistance of all types but not equally from about 0.3 unit/ml to 0.6 to 10 units/ml.

Comment. The limit of growth tolerance of *G. tetragena* strain *6007,* to penicillin in amounts more than .002 unit/ml in our hands was much lower than that reported by Mock and Wynne who obtained growth on agar containing 2 units/ml(3). Resistance of a different strain to 125 units/ml was reported in clinical studies(4). In other tests, the tolerance of different strains ranged from .016 to .06 unit ml(5.) Evidently, the resistance of strains of *G. tetragena,* like certain other bacteria, varies greatly and perhaps differences in technic may account for some of the wide range. Mock and Wynne failed to induce increased resistance to penicillin, and succeeded to do so against streptomycin from 0.5 unit/ml, initially to as much as 1000 units/ml after 4 serial transfers. In our experiments, induced resistance rose at most from 0.3 unit/ml to about 10 units/ml and to that degree only with the yellow and mucoid-pink variants.

We also were unable to increase the resistance to penicillin of the white type or of any of the variants derived from the 2 strains tested. After 9 and 18 passages on streptomycin-agar, resistance of each variant increased but to different degrees, greatest with

the yellow, mucoid-white and mucoid-pink types, least with the white and brown. There was no evidence of spontaneous or induced variation to account for significantly increased resistance or for the survival of one variant over another. However, Leidy and Alexander found that the incidence of resistant variants in cultures of *H. influenzae* varied from 1 to 1 billion to 1 to 14 billions organisms(6). It is possible in our experiments that some cocci were missed which might have had or developed resistance to penicillin or to streptomycin as great as that reported by others.

Our observations lend support to views (a) and (b) mentioned previously to the extent that variant forms of the strains of *G. tetragena* tested can acquire more resistance to an antimicrobial, streptomycin in this case, than the parent white type. There was, however, no evidence that exposure to antimicrobials induced variation any more than other adverse growth conditions did(2). The ability of *G. tetragena* to dissociate, apparently by chance, may indeed provide means for its survival *in vitro* if any new variant emerges and is able to grow better than the original form in the presence of an antimicrobial.

The results of the tests favor view (c) as well. A gradual adaptation, without visible colonial changes, also took place allowing the development of unequal grades of resistance of each variant independently.

In general, the observations agreed with those previously reported wherein dissociation, more or less by chance, provided variants hardier than the parent cocci(2). In addition, the gradual adaptive ability demonstrated against streptomycin suggests that either or both changes may enable *G. tetragena* to continue growth in certain unfavorable conditions. The magnitude of resistance developed by either method may be of therapeutic significance. So far as is known, infection in man is caused by *G. tetragena* in its S-white colony type. The white type as noted here acquired resistance without evident variation and this may account for the development of resistance as observed clinically(4). Type or phase variation rarely is detected during disease(7).

Conclusion. G. tetragena, strains *6007* and *10875,* failed to acquire resistance to penicillin after repeated transfers in or on media containing it, nor did the procedure induce visible variation or reversion of the usual dissociant forms. Growth on streptomycin-agar increased the tolerance of each variant to 5 to 30 times the original amount of streptomycin which may be of clinical significance. There was no evidence that variant forms better fitted than the original one to grow under new adverse circumstances were either induced or purposefully developed. The change seemed to be a gradual adaptive one.

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Effect of Clipping on Body Temperature of Restrained and Nonrestrained Rats Exposed to Cold. (23036)

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Small, lightly restrained mammals become rapidly and progressively hypothermic in a cold environment in which nonrestrained animals maintain normal temperatures(1). From observations of many hundred rats, it has become obvious to us that piloerection is less in restrained hypothermic rats than in nonrestrained controls. It was therefore reasoned that shaving restrained animals (where the less erected hair offers poor insulation) should not cause as great a fall in body temperature as in nonrestrained, usually normothermic, animals. If this were true, poor piloerection could be regarded as one cause of the hypothermia caused by restraint.

Methods and materials. Forty adult (225-275 g) male Sprague-Dawley rats were divided into 4 experimental groups: 10 nonrestrained, nonclipped; 10 nonrestrained, clipped; 10 restrained, nonclipped; and 10 restrained clipped animals. Restraint was produced by maintenance in loose fitting wire mesh cylinders flattened on the underside and closed at the ends. Clipping was as complete as possible with a conventional animal clipper. Exposures were at 3° ± 2°C and were 2½ hr in length unless falling body temperature and death terminated the experiments earlier. Colonic temperatures in both control and restrained animals were obtained from a Thermister inserted for each measurement 7 cm into the rectum. The body temperature determinations were not made on a definite time schedule. Thus, in Fig. 1, all the readings in the 0-25 min interval, 26-50 min interval, etc. have been averaged.

Results. As observed in Fig. 1, removing hair from restrained animals resulted in a much lesser additional body temperature fall than a similar treatment of nonrestrained animals. It therefore appears that the restrained rat becomes hypothermic at least partly as the result of an increased rate of heat loss per unit body surface exposure. That this is not the sole cause of hypothermia seems clear from the observation (Fig. 1) that restrained clipped animals became more hypothermic than nonrestrained clipped animals.

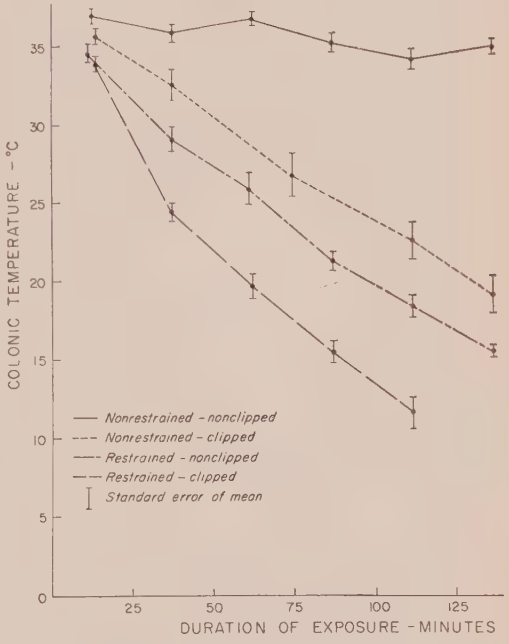

FIG. 1. Effect of clipping on body temp. of restrained and nonrestrained rats at 3° ± 2°C. Body temp. determinations were as frequent as possible and therefore temporally random. For graphic presentations, data have been grouped by time intervals, 25 min. apart. The 51-100 min. interval is presented as a single point in the nonrestrained clipped animals. The precipitous body temp. fall of restrained clipped animals resulted in termination of exposures at 125 min.

Increased convective heat loss in the restrained struggling rat may be another factor in the hypothermia production. Also, the body temperatures of both clipped nonrestrained and clipped restrained animals fell the same per cent of the range between the temperature of the respective nonclipped controls and the environmental temperature. Thus, although these experiments are taken

as presumptive evidence that inadequate piloerection is one factor in the production of restraint hypothermia, other mechanisms cannot be excluded.

Summary. Clipping of restrained rats resulted in a much lesser additional body temperature fall than a similar treatment of nonrestrained animals. This is interpreted as supporting the hypothesis that the less erected hair of the restrained animal results in a greater rate of heat loss than the fully erected hair of the nonrestrained animal.

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Cytology and Hormone Content of Rat Pituitary Glands Following Adrenalectomy. (23037)

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The problem of the cellular source of corticotropin has been investigated most frequently in experimental animals by studying pituitary cytology after alteration of normal pituitary-adrenal gland relationship, accomplished by adrenalectomy, administration of pituitary extracts, corticotropin or adrenal cortical hormone. Because these experimental procedures influence also the secretion of hormones other than corticotropin(1-3), changes which occur in pituitary cytology should be interpreted not only in terms of the alteration produced in the secretion of corticotropin, but with respect to other pituitary hormones as well. The present investigation is an attempt to localize the site of corticotropin production by correlating cytological changes in the rat's pituitary gland following adrenalectomy with alterations in content of several of the hormones elaborated by this gland.

Materials and methods. Sprague-Dawley male rats weighing 200-250 g were bilaterally adrenalectomized and maintained on saline drinking water for 28, 56 and 100 days. Pituitary glands were investigated histologically with Masson trichrome stain, the PAS, acid hematin and Bodian protargol methods as described previously(4,5). An average of 1585 cells were counted in each gland in making differential cell counts from both trichrome- and protargol-stained sections. Pituitaries of control rats and animals adrenalectomized for 100 days were assayed for their relative hormone content. Glands used for these analyses were either homogenized in saline and assayed the following day, or they were frozen in dry ice-acetone or liquid nitrogen, lyophilized and stored in a deep freezer. In separate assays, using 22- to 26-day-old hypophysectomized female rats as recipient test animals, the relative content of gonadotropins, thyrotropin and growth hormone was estimated by comparing ovarian and uterine weight and histology, thyroid weight and I^{131} uptake, and epiphyseal plate response(6) respectively. Recipient animals received a total of 5 or 10 mg of pituitary tissue (wet weight) injected in divided doses over a 4-day period. Pituitary thyrotropin content of 6 control and 6 adrenalectomized rats was assayed also according to Levey *et al.*(7). One dosage level was employed, an amount of donor pituitary tissue equivalent to 1/50th of the gland weight, was administered intravenously to 6 test animals for each pituitary gland assayed. Corticotropin content of the pituitary was measured by the ascorbic acid depletion test(8) with 3 dosage levels, according to Halmi and Bogdanove(9), being employed in each assay.

Results. Weight and cytology of pituitary glands. Pituitary glands of 193 adrenalectomized and 184 control animals were weighed, the mean weights 28, 56 and 100 days after adrenalectomy being 8.7, 9.3 and 11.1 mg respectively. Corresponding weights of glands

TABLE I. Relative Percent of Cell Types in Pituitary Glands of Adrenalectomized Male Rats.

Group	Acidophils	β-Basophils	δ-Basophils	Chromophobes	Argyrophils*
Control	39.0 ± 1.5†	2.6 ± .3	4.2 ± .3	54.2 ± 2.6	5.3 ± .4
Adrx- 28	32.1 ± 2.1	2.6 ± .1	3.6 ± .3	61.7 ± 3.4	4.2 ± .4
- 56	38.6 ± 1.7	1.8 ± .2	3.8 ± .1	56.4 ± 2.1	3.3 ± .3
-100	40.8 ± 1.3	1.1 ± .1	4.0 ± .3	55.1 ± 4.0	2.9 ± .2

* Determined from FAA-Protargol preparations; others from Bouin-Masson preparations.
† Determinations made from 8 pituitary glands in each group. Mean ± stand. dev.

TABLE II. Relative Gonadotropic, Thyrotropic and Growth Hormone Activity of Pituitary Glands of Control and Adrenalectomized Rats.

	Recipients (22-26-day-old hypophysectomized female rats)								
		Gonadotropins			Thyrotropin			Growth hormone	
Donor	Dose,* mg donor pituitary	No.	Ovary, mg	Uterus, mg	No.	Thyroid, mg	I^{131}, % A.D.†	No.	Epiphyseal plate, μ
Control		22	13.3 ± 2.6‖	33.8 ± 8.1	8	3.8 ± .20	.05 ± .002	13	203 ± 11
	5				6	5.25 ± .22	1.72 ± .25	6	324 ± 13
	10	10	28.2 ± 4.2	126 ± 12	8	6.61 ± .61	2.83 ± .45	6	373 ± 10
Adrx-100‡	5				7	4.10 ± .37	1.21 ± .35	5	327 ± 9
	10	14	19.8 ± 2.8	96.5 ± 9.2	8	4.84 ± .40	1.73 ± .10	7	352 ± 13
	100 μg GH§							6	302 ± 9
	200 " "							8	348 ± 11

* Administered in divided portions for 4 days. † A.D. = Administered dose of I^{131}; 3 μc, 4 hr prior to autopsy. ‡ Adrx-100 = Adrenalectomized 100 days. § GH = Beef growth hormone, Armour, Lot No. 285-183. ‖ Mean ± stand. dev.

from control animals were 8.8, 9.1 and 10.1 mg. The relative percentages of cell types in pituitary glands of control and adrenalectomized rats are summarized in Table I. In Masson-stained sections, acidophils did not exhibit notable alteration in relative number or appearance following adrenalectomy. Beta basophils, identified by their staining with aldehyde fuchsin, were reduced at 100 days after adrenalectomy to approximately one-half the number present in control glands. Delta basophils, whose cytoplasmic granules are colored by the light green component of the Masson stain, were not notably altered in number. At 56 days after adrenalectomy, hypertrophy of the delta basophils was noticeable, and there was present at 100 days some degree of vacuolization of these cells. Adrenalectomy produced a slight but consistent decrease in number of PAS-positive basophils, although the staining reaction of individual cells varied considerably. Staining of acidophils by acid hematin for phospholipids was not altered from that observed in control glands. The relative number of argyrophilic cells declined progressively following adrenalectomy and at 100 days after operation was reduced to approximately one-half the number present in control glands (Table I).

Hormone content of pituitary glands. Growth hormone. Estimation of pituitary growth hormone content of control animals and animals adrenalectomized for 100 days revealed no difference between the two groups, injection of 5 or 10 mg of control or experimental pituitary glands producing similar epiphyseal plate responses (Table II). Daily injection of 100 or 200 μg of growth hormone* produced epiphyseal plate responses approximately equal to that produced by administration of these amounts of pituitary tissue respectively.

Thyrotropin. Pituitary thyrotropic hormone content, as measured by thyroid weight and radioiodine uptake in hypophysectomized test animals, was decreased 100 days after adrenalectomy (Table II). I^{131} uptake produced by glands of adrenalectomized rats was significantly less than that produced by

* Beef growth hormone, Armour, Lot No. 285-183, kindly supplied by Dr. Sanford L. Steelman.

TABLE III. Corticotropin Content in Pituitary Glands of Adrenalectomized Male Rats, Assayed by the Adrenal Ascorbic Acid Depletion Test.

Groups	Mean response at indicated dose 1.25*	.62	.31	Slope of log-dose response curve	M	Antilog of M	SM	Limits of error, P = .95
Control (6)†	30.1§ (26.0–36.2)‖	20.2 (7.5–28.2)	15.0 (8.1–24.2)	25.1	.5611	3.640	.098	2.91–4.56
Adrx-28‡ (8)	39.4 (33.8–50.4)	35.0 (22.0–38.8)	28.2 (18.4–35.3)	19.0				
Control (6)	28.7 (18.4–35.0)	18.9 (11.3–30.1)	13.3 (2.5–23.3)	25.6	.5372	3.445	.091	2.79–4.25
Adrx-56 (12)	42.4 (33.0–44.6)	32.6 (15.2–43.5)	27.1 (14.2–33.0)	25.5				
Control (6)	28.2 (20.3–32.5)	19.8 (12.0–33.1)	11.8 (3.0–20.3)	29.2	.3164	2.072	.086	1.70–2.53
Adrx-100 (9)	35.3 (29.4–41.3)	30.6 (18.6–34.4)	20.3 (12.5–30.2)	27.5				

* Dose = % of donor pituitary/100 g recipient. † No. of pooled pituitaries. ‡ Adrx = Adrenalectomized 28, 56 and 100 days. § Response = Mean of difference between ascorbic acid in left and right adrenal of recipients, expressed in % of concentration in left adrenal. ‖ Range, with 6 test animals in each instance.

control pituitaries ($P = <0.05$ at both 5- and 10-mg dosage levels). The mean thyrotropin content of 6 control pituitary glands assayed by the method of Levey *et al.*(7) was 83 mμ/mg, while that of 6 animals adrenalectomized for 100 days was 39 mμ/mg.

Gonadotropin. Pituitary glands of adrenalectomized rats produced significantly less ovarian weight ($P = <0.01$) and less uterine weight ($P = <0.05$) than did those of corresponding control animals (Table II). Ovarian histology of recipient test animals revealed no qualitative difference in action of gonadotropins from control and experimental glands, both stimulating follicular growth without corpora lutea formation and repairing the interstitial tissue ("cartwheel" chromatin of the nuclei) to approximately the same degree.

Corticotropin. Adrenalectomy led to a 2- to 3½-fold increase in pituitary corticotropin content (Table III). After removal of adrenal glands, there occurs an initial drop in pituitary corticotropin content(10), followed by a rise to normal content in 7 days(11), and continued increase thereafter to 2-3 times the normal content 21 days after adrenalectomy(12). Results indicate that maximum corticotropin content is reached at 28 or 56 days after adrenalectomy and may decline somewhat at 100 days.

Discussion. Results of our study offer several correlations between changes in cytology and hormone content following adrenalectomy. The observations of an apparent normal growth hormone content of the pituitary gland of adrenalectomized rats correlated with a normal relative number of acidophils may add additional evidence in favor of this cell as the source of growth hormone. Because other pituitary hormones (corticotropin, thyrotropin) may exert antagonistic or synergistic effects upon growth hormone in this method of assay, some caution must be exercised in interpreting the results produced by administration of whole pituitary extracts.

The observation of a decrease in thyrotropin store of the pituitaries of adrenalectomized animals together with a fall in number of aldehyde-fuchsin staining beta basophils, adds to the increasing evidence that this cell is the secretor of thyrotropin.

The PAS-demonstrable glycoprotein granules of delta basophils are considerd to represent the gonadotropic hormones(13). Knigge(5) has presented evidence indicating that fluctuations in gonadotropic hormone content of the pituitary are: (a) reflected also by changes in certain silver-staining (argyrophilic) cells in the pars distalis; and (b) that

these "argyrophilic cells" are probably identical with delta basophils, stained by a procedure in which certain cytoplasmic granules are blackened with silver. With the protargol silver method, the reduction in silver-staining granules of delta basophils after adrenalectomy correlated with the observed fall in gonadotropin content of the pituitary.

In considering the cellular source of corticotropin, the following observations are pertinent: (1) corticotropin was the only hormone whose content was increased in the pituitary after adrenalectomy; and (2) delta basophils, as revealed by the Masson method, were the only cells which showed cytological signs of increased activity. Increased titers of corticotropin are present in blood following adrenalectomy (11), suggesting that the pituitary is secreting as well as storing more of this hormone. These facts, together with the other data presented in this study, contribute to the conclusion that the delta basophil is the site of corticotropin production. It is felt, however, that the cytoplasmic granules of delta basophils, as revealed in Masson preparations, do not *per se* reflect corticotropin content of the cell. In acute or short-term experiments, no satisfactory single staining procedure is currently available for detecting changes in activity of delta basophils as related to corticotropin secretion.

Summary. 1. Adrenalectomy produced no significant change in weight of rat's pituitary gland. Cytologically, beta basophils decreased in number progressively while acidophils were not altered in number or appearance. The relative per cent of delta basophils was unaffected, but these cells were hypertrophied and exhibited some vacuolation 56 and 100 days after adrenalectomy. The silver-staining capacity of delta basophils, as revealed with protargol, was markedly reduced. 2. Gonadotropic and thyrotropic hormone contents of pituitary were decreased after adrenalectomy, while the corticotropin content was elevated 2- to 3-fold. No difference in growth hormone content was found when whole pituitaries were analyzed by the epiphyseal plate test. 3. Correlations between pituitary cytology and hormone content of the gland suggest that the delta basophil is the source of corticotropin.

Appreciation is expressed to Dr. Harold Levey for the thyrotropin assay according to Levey, Cheever and Roberts (1956).

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Effect of Vit. E-Deficiency on Free Amino Acids of Various Rabbit Tissues.* (23038)

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Roderuck(1) demonstrated that the glutamine content of skeletal muscle of dystrophic guinea pigs was reduced from that of muscle of control animals, whereas the content of non-glutamine amino acids was unchanged. Vit. E-deficient rabbits showed less marked differences. Tallan(2) reported that in severe dystrophic rabbit, the levels of most free amino acids in muscle extracts were elevated except for concentration of glycine which was markedly reduced, even in early stages of the disease. In contrast to this finding, Dinning *et al.*(3) were unable to show any consistent effect of vit. E-deficiency upon concentration of free glycine of muscle or liver. It seemed, therefore, desirable to resolve these differences as well as to determine what effect, if any, the dystrophic condition had on concentration of free amino acids of tissue other than muscle. Since it may be presumed that free amino acids constitute a major source of the protein building blocks of tissue, the concentration of free amino acids of atrophied muscle, as well as that of supposedly unaltered organ tissue from a dystrophied animal, should be of considerable interest. The studies here reported describe the concentration of 9 free amino acids in liver, kidney, heart, spleen, brain, and muscle of normal and vit. E-deficient rabbits.

Methods. Four male rabbits weighing 1250 g to 1475 g were fed the dystrophy producing diet of Goettsch and Pappenheimer(4) while 2 litter mates consumed an adequate diet. Creatine was determined by the method of Folin(5) and it was noted that those animals on the deficient diet exhibited a slight creatinuria within 3 weeks, while controls showed none. The onset of dystrophy was considered to have started when creatine excretion rose rapidly, consequently when this point was reached, the 6 animals were sacrificed. Two animals showed a severe dystrophy as evidenced by respiratory difficulty, and inability to right themselves after being placed on their sides. The other 2 deficient animals had less pronounced outward symptoms as well as lower degree of creatinuria. Thus these two sets of deficient animals would compare with early and severe dystrophy animals described by Tallan(2). As soon as animals were sacrificed, samples of tissue were immediately homogenized with a Teflon pestle in distilled water. Aliquots of the homogenate were removed to determine dry weight of tissue, and the remainder was treated with 10% trichloroacetic acid to remove the proteins. After removal of acid with ether, the resulting solution was concentrated *in vacuo*. Quantitative determination of the amino acids was then made by Dowex-50 chromatography as previously described(6).

Results. Distribution of free amino acids in various tissues of normal and dystrophic rabbits is shown in Table I. The values are expressed as mM/100 g dry tissue, and represent the average of tissues from 2 rabbits, except where noted. The results listed in Table I show that muscle and heart of severely dystrophic animals had a general increase in concentration of free amino acids with the exception of taurine and glycine. The same results were also evident in mildly dystrophic animals although the differences were not as pronounced. On the contrary, Table I demonstrates that brain homogenates of severely dystrophic animals exhibited a general decrease in concentration of free amino acids, especially of serine. Only ethanolamine phosphate and glutamic acid displayed a slight increase. This same trend was also evident in the brain of mildly dystrophic animals. Kidney homogenate of dystrophic animals likewise evinced a decrease in certain amino acids, *viz*: ethanolamine phosphate, serine,

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TABLE I. Free Amino Acid Concentrations of Tissues from Normal and Vit. E-Deficient Rabbits. Values expressed as mM/100 g dry tissue and probable error of the mean.

Amino acid	Muscle		Heart		Brain		Kidney		Spleen		Liver	
	Normal	Vit. E-def.	Normal	Vit. E-def.	Normal	Vit. E-def.	Normal	Vit. E-def.	Normal	Vit. E-def.	Normal	Vit. E-def.
Ethanolamine phosphate	.13 ± .04	.31 ± .03	.39 ± .02	.41 ± .03	1.21 ± .08*	1.43 ± .09	2.92 ±1.00	1.90 ± .03	2.98 ± .10	2.80 ± .41	.35 ± .06	.81 ±.06
Taurine	.74 ± .12	.43 ± .19	6.72 ±1.4	6.6 ±1.4	1.08 ± .04	1.04 ± .02	1.51 ± .39	1.75 ± .03	2.74 ± .14	2.83 ± .24	.24 ± .05	.63 ±.24
Aspartic acid	.10 ± .00	.19 ± .04	.53 ± .09	.56 ± .15	1.96 ± .24	1.53 ± .08	.65 ± .37	.70 ± .03	1.22 ± .05	1.17 ± .07	.72 ± .28	.76 ±.06
Threonine	.11 ± .01	.32 ± .06	.11 ± .01	.20 ± .02	.23 ± .00	.18 ± .01	.21 ± .13	.46 ± .01	.44 ± .03	.57 ± .05	.16 ± .00	.15 ±.00
Serine	.48 ± .07	.79 ± .20	.46 ± .04	.72 ± .18	2.32 ± .60	.93 ± .12	1.74*	.90 ± .02	1.14 ± .11	1.19 ± .06	.75 ± .06	.45 ±.07
Glutamic acid	.56 ± .12	.67 ± .08	1.30 ± .06	1.44 ± .11	3.54 ± .43	4.05 ± .78	2.94*	2.47 ± .06	3.73 ± .15	4.02 ± .13	1.29*	1.41 ±.27
Proline	.00 ± .00	.98 ± .15	.32 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	2.04*	1.73 ± .04	1.21 ± .20	1.56 ± .11	.60*	.81 ±.10
Glycine	1.77 ± .16	1.04 ± .13	.65 ± .09	.34 ± .05	.86 ± .03	.72 ± .05	4.58*	1.96 ± .03	2.61 ± .16	1.83 ± .08	1.50*	1.77 ±.02
Alanine	.66 ± .16	1.10 ± .22	1.86 ± .04	2.31 ± .30	.51 ± .00	.46 ± .05	1.58*	1.13 ± .01	.94 ± .04	1.26 ± .01	1.28*	.93 ±.09

* Single determination.

alanine, and glycine. Only threonine showed a distinct increase. Table I indicates that spleen homogenate showed a general increase in concentration of most amino acids, only glycine exhibited a significant decrease. Liver homogenate manifested an increase in ethanolamine phosphate and taurine, with only alanine and serine showing pronounced decreases.

Discussion. Perhaps the most important point to be made from these experiments is that vit. E-deficiency in rabbits led to a marked reduction in free glycine in 5 of the 6 tissues examined. Only liver showed a slight increase in glycine. Tallan found similar results in dystrophic muscle, *viz.* a decrease in free glycine of muscle approximating 46% as compared to our value of 42%. These results therefore differ from Dinning *et al.*(3) who demonstrated a slight increase in glycine of vit. E-deficient rabbit muscle. Serine, a known precursor of glycine, was markedly reduced in the kidney, liver, and especially the brain.

It has been shown(7) that there is an increased turnover rate of nucleic acids in vit. E-deficient animals. Dinning *et al.*(3) have also demonstrated that vit. E-deficiency led to greatly increased incorporation of formate-C^{14} into nucleic acid purines, but resulted in a decreased incorporation of glycine-(1)-C^{14} into purines. These results were explained on the basis of an exchange reaction of the 2 position of the purine ring without complete degradation of the molecule. Inasmuch as only the alpha carbon of glycine has been shown to be a precursor of formyl fragments, the work of Dinning *et al.*(3) does not exclude the possibility that glycine is contributing its methylene carbon to the 2 position of the purine ring since the C^{14} of their injected glycine molecule was not located in the methylene portion.

Our data suggest that perhaps glycine, as well as its precursor serine, may be acting as the main source of the formyl fragment. This would tend to deplete these two amino acids from the various metabolic pools. The possibility also exists that there is an accelerated rate of protein breakdown to furnish the

needed glycine. It is of interest that Weinstock *et al.*(8) have recently shown proteolytic activity of muscle from vit. E-deficient rabbits to be increased. This could account for the observed increase in other free amino acids of dystrophic muscle.

On the basis of these observations, it is concluded that vit. E-deficiency in rabbits is characterized by a distinct pattern change in the free amino acid levels of various tissues. The decrease in glycine suggests that it is in some unknown way implicated in the dystrophic state.

Summary. 1. Concentration of free amino acids from 6 tissues of normal and vit. E-deficient rabbits has been determined by Dowex-50 chromatography. 2. There was a general increase in free amino acids of muscle, heart, spleen, and liver of dystrophic animals. Only glycine and serine were markedly reduced. 3. Kidney and especially brain showed a general decrease in free amino acids. 4. Concentration of glycine was more consistently reduced in various tissues than other amino acids.

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Comparative Androgenic and Anabolic Effects of Several Steroids. (23039)

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The common androgens, testosterone propionate and methyl testosterone, produce anabolic as well as androgenic effects(1,2). In man, the androgenic activity frequently limits the use of these compounds as anabolic agents. Attempts to increase relative anabolic potency have led to development of methyl androstenediol(3) (Methostan, Methandriol), androstanolone(4) (Neodrol, Stanlone) and 17 - ethyl - 19 - nortestosterone(5) (Nilevar). The present study was underaken to compare the anabolic and androgenic potencies of these newer agents with testosterone propionate, methyl testosterone and unesterified testosterone.

Materials and methods. The method used was that of Eisenberg and Gordan(6). The rats were castrated at 23-25 days of age. Starting 3 weeks later, the compounds dissolved in corn oil, were administered intramuscularly daily for 7 days. Testosterone, testosterone propionate and methyl testosterone were compared with methyl androstenediol, androstanolone and 17-ethyl-19-nortestosterone. Eight rats were used at each dose level and total doses of 0.02 to 5 mg per rat were administered. A total of 432 rats was used; of these, 88 served as oil-treated controls. Gains in body weight, as well as terminal weights of the seminal vesicle and ventral prostate glands and levator ani muscles, were examined and compared with these parameters in controls autopsied at the same time. The regression lines shown in Fig. 1 were calculated by the method of least squares using only those values at and above the minimal effective range.

Results. Anabolic effects. Increase in weight of the levator ani muscle was used as a measure of anabolic activity. In Fig. 1, the differences between weight of this muscle in the treated animals and in controls run at the same time are plotted against the log of the dose. Marked anabolic responses were

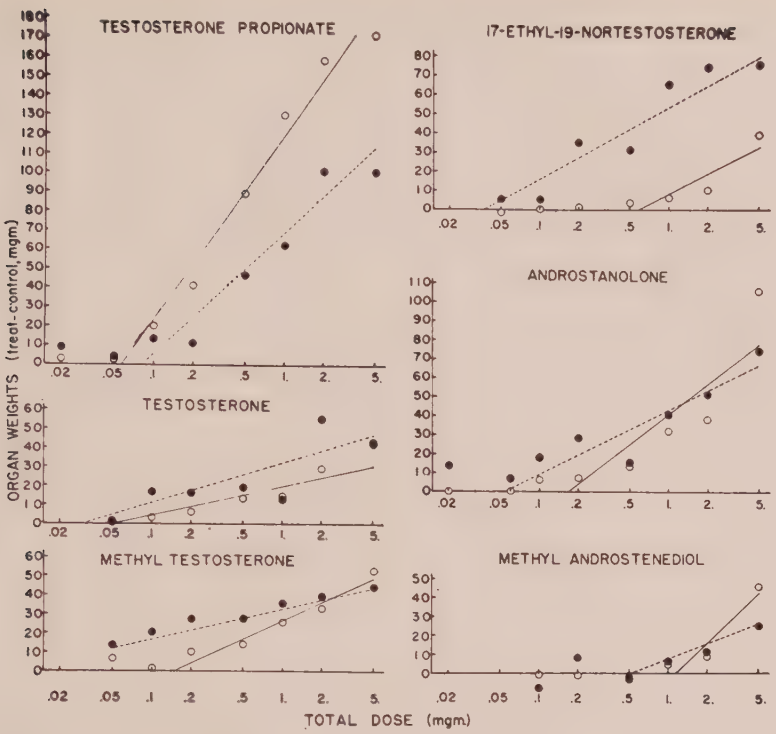

FIG. 1. Responses of seminal vesicles (open circles) and levator ani muscles (solid circles) of castrated male rats to graded doses (log scale) of various steroids. Each point is the difference between avg organ weights of a group of 8 treated animals and controls run simultaneously. Regression lines were calculated by the method of least squares from those values which showed a significant increase over the controls. Solid lines indicate androgenic potency while broken lines represent anabolic effects.

obtained with testosterone propionate, 17-ethyl-19-nortestosterone, and androstanolone while curves for testosterone and methyl testosterone exhibited a more gentle slope. Methyl androstenediol was much less active than the other compounds.

Body weight. Overall gain for the 88 control animals was 36 g during the week of treatment. Groups of 8 animals each showed average gains ranging from 31 to 41 g. In general, doses of these steroids which caused significant increases in levator ani muscle weight also caused gains in body weight in excess of the gains seen in the controls. With 8 animals per group these increased gains were not always statistically greater than the controls. The pooled results at anabolic dose levels are presented in Table I. Significantly increased gains in body weight were obtained with testosterone, testosterone propionate, androstanolone and 17-ethyl-19-nortestosterone. Although the table does not show a significant increase with methyl testosterone, at the 5 mg dose level the increase obtained with this compound was significant. The data on methyl androstenediol are from a single group of rats and no increased gain was obtained with this compound at the doses used.

Androgenic activity. Relative androgenic potencies of these steroids are also depicted in Fig. 1. Testosterone propionate was by far the most potent androgen studied and its androgenic effect, as measured by increase in seminal vesicle weight, far exceeded anabolic response, as indicated by increase in the weight of the levator ani muscle. With testosterone, methyl testosterone, and androstanolone androgenic and anabolic responses to a given dose, as shown by these parameters, were reasonably close together. However, it will be noted that methyl androstenediol showed little androgenic or anabolic activity

TABLE I. Effects of Various Steroids on Gains in Body Weight in Castrated Rats.

	Dose range, mg	No. of rats	Mean body wt, g	Mean diff. from control, g	t
Control		88	35.58		
Testosterone propionate	.2-5.	40	41.90	6.32 ± 1.06*	5.96
Testosterone	1. -5.	23	42.43	6.85 ± 1.31	5.23
Methyl testosterone	1. -5.	24	37.54	1.96 ± 1.28	1.53
Methyl androstenediol	5.	8	34.25	−1.33 ± 2.06	− .65
Androstanolone	.5-5.	31	42.51	6.93 ± 1.17	5.92
17-Ethyl-19-nortestosterone	.2-5.	40	39.42	3.84 ± 1.06	3.62

* ± stand. error.

TABLE II. Androgenic and Anabolic Responses to Various Steroids in Castrated Rats.

Compound	Total dose (mg) required to produce 100% increase in sem. ves. wt	Total dose (mg) required to produce 50% increase in lev. ani wt	Potency relative to testosterone propionate (%) Androgenic	Potency relative to testosterone propionate (%) Anabolic	Potency ratio, anabolic/androgenic
Testosterone propionate	.073	.26	100	100	1.00
Testosterone	.21	1.00	35	26	.74
Methyl testosterone	.30	1.00	24	26	1.08
Methyl androstenediol	1.6	3.65	5	7	1.58
Androstanolone	.24	.45	30	58	1.93
17-Ethyl-19-nortestosterone	1.10	.25	7	104	14.85

while 17-ethyl-19-nortestosterone had marked anabolic activity combined with a low androgenic potency. Increases in the weights of the ventral prostate glands were similar in degree to the changes in seminal vesicle weights so the data are not presented here. These effects on the ventral prostate confirm the conclusions regarding relative androgenic potency of these steroids.

Discussion. Hershberger *et al.*(7) proposed the anabolic ratio as a measure of degree of separation of anabolic and androgenic effects. They used smaller rats and calculated androgenic effect from the weight of the ventral prostate gland. With our older rats it was observed that this ratio was fairly constant only when the organ responses were marked, that is, when increase over the controls was at least 20 mg.

From Fig. 1 the doses required to produce a 100% increase in seminal vesicle weight (to 20.2 mg) and a 50% increase in levator ani weight (to 97.0 mg) were calculated. The potency relative to testosterone propionate at these points was determined (Table II). From these data, the anabolic/androgenic ratio was calculated. Since the slopes of the various lines differed, the calculated ratios in Table II hold only for this set of conditions. Slight separation of anabolic and androgenic effects was obtained with androstanolone, which gave a ratio of 1.9:1 and methyl-androstenediol with a ratio of 1.6:1, while 17-ethyl-19-nortestosterone showed a much greater separation with a ratio of 15:1.

Gordan *et al.*(8) reported that methyl androstenediol had an anabolic potency equivalent to that of methyl testosterone but that it produced androgenic effects only at excessively high doses. Hershberger *et al.*(7) found this compound to have only 5% the anabolic potency of testosterone propionate with no separation of activities. Barnes *et al.*(9), using younger rats than were studied in the current report, and treating for a longer period, found that methyl androstenediol had an androgenic potency of 2.1% and a myotrophic potency of 2.2% compared to testosterone propionate giving a ratio of 1. From our results corresponding values of 7.1 and 4.5% were obtained, with a ratio of 1.6. These results agree with those of Barnes *et al.* rather than with those of Gordan *et al.* Henderson and Weinberg(10) found that when administered subcutaneously in rats, using the seminal vesicles as the end point this compound was about ⅓ as androgenic as testosterone, but its effect on the capon comb, after

parenteral administration, was only 2% that of testosterone.

Kochakian(2) reported that androstanolone was about as androgenic as testosterone propionate in castrated mice. He used a very limited number of doses. The renotropic effects of the two compounds were also similar. Barnes *et al.*(9), using rats, calculated a myotrophic potency of 21.8% and an androgenic potency of 13.2%, thus giving a ratio of 1.6. Again our corresponding figures are slightly higher or 58% and 30% respectively, but the ratio is similar, 1.9. Barnes *et al.* also reported that the cyclopentyl-propionate of 19-nortestosterone was about 1.5 times as potent as testosterone propionate as an anabolic agent and ascribed to it an anabolic/androgenic ratio of 9.3. Our results show 17-ethyl-19-nortestosterone to have a myotrophic potency of 104% and an androgenic potency of only 7% giving an anabolic/androgenic ratio of 15, thus showing the widest separation of effect hitherto reported.

Summary. The androgenic and anabolic effects of testosterone, testosterone propionate, methyl testosterone, methyl androstenediol, androstanolone and 17-ethyl-19-nortestosterone (Nilevar) were compared. The greatest androgenic responses were obtained with testosterone propionate while the most marked anabolic effects were observed with either testosterone propionate or 17-ethyl-19-nortestosterone. Compared to testosterone propionate, methyl androstenediol and androstanolone showed a small increase in the anabolic: androgenic potency ratio but a much more marked increase was observed with 17-ethyl-19-nortestosterone.

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Effects of Age, Castration and/or Steroids upon Hematocrit in Male Rats.* (23040)

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The effects of age, castration and steroids on the blood of male rats have been studied previously. It has been reported(1) that hemoglobin content rose from 50th to 150th day of life. Several investigators(2-4) have shown that gonadectomy causes mild, transient anemia; these workers and others(2-7) reported that this anemia could be repaired by subcutaneous injections of androgen. Estrogen has been reported to have a moderate (4) or no(7) effect on male rat blood. A recent review article discusses erythropoietic stimuli in additional species(8).

The micro-method of Strumia, Sample and Hart(9) permits hematocrit determinations with very small amounts of blood, thus allowing multiple determinations on a single ani-

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TABLE I. Group Organization and Data.

Group No.	Treatment	Age (days)	No. of animals	Hematocrit
I	Intact animals, untreated	54- 73 m 65 *	22	45.2 ± .4†
II	*Idem*	74-107 m 83.5	22	49.3 ± .5
III	Castrates otherwise untreated	74-102 m 85.9	22	45.3 ± .6
IVa	Self-controls, before castration	60- 87 m 73	16	46.3 ± .7
IVb	" , 14 days after castration	74-101 m 87	16	42.4 ± 1.0
V	Castrates inj. with sesame oil alone	77-102 m 87.7	8	47.3 ± 1.7
VI	Castrates inj. with sesame oil containing 500 μg testosterone propionate	75-108 m 90.1	10	50.1 ± .6
VII	Castrates inj. with 500 μg aqueous testosterone	89- 97 m 92.9	5	49.0 ± 1.6
VIII	Intact, inj. with sesame oil alone	77-102 m 84.4	7	51.6 ± 1.3
IX	Intact, inj. with sesame oil containing 500 μg testosterone propionate	76- 84 m 81.2	10	51.3 ± 1.4
X	Intact, inj. with sesame oil containing 50 μg estradiol benzoate	76- 90 m 82.1	12	48.5 ± 1.6
XI	Castrates inj. with sesame oil containing 50 μg estradiol benzoate	76-101 m 86.7	7	46.8 ± .6
XII	Neonatally castrated animals, otherwise untreated	84-111 m 96	15	50.9 ± 1.0

* m = mean. † Stand. error of mean.

mal. Using this method we investigated the effects of age, castration and/or steroids upon hematocrits of male rats.

Materials and methods. Male rats of locally inbred Osborne-Mendel strain were utilized. Hematocrits were done on blood from cut terminal tail veins or small femoral tributaries. The capillary tubes were held nearly horizontal and one end placed in freely-flowing blood. One drop of blood fills 4 tubes, and very little blood was lost in determining an hematocrit. Tubes were centrifuged at 17,500 rpm for 2 minutes and read on a Drummond microhematocrit reader. The average of a set of 2-6 individual capillary tubes was considered to be the *hematocrit;* 140 hematocrits were thus calculated from 530 individual tubes. There was very little spread among individual tube values; in 18 sets the extreme variation was more than 2.5 points, in 75 sets the extreme variation was from 2.5 to 1, and in 47 sets the extreme variation was 1 point or less. Twelve groups of animals were studied, based upon age and/or treatment. The groups included intact rats of different ages, animals castrated at different ages, intact and castrated animals injected with sesame oil (S.O.) alone, or with S.O. containing hormones,‡ and castrated animals injected with a water-soluble testosterone.§ Litter mates were placed in different categories when possible. In a group called *self-controls,* hematocrits were determined at castration and again 14 days later. Animals castrated within first 24 hours of life are called *neonatal castrates.* Those castrated as adults are referred to as *castrates;* in these 2 weeks elapsed between gonadectomy and onset of injections. When injections were made, 0.5 cc of fluid was given subcutaneously on 7 consecutive days. Specific treatment of each group is indicated in Table I. Mean hematocrits were calculated for each group, comparisons made between groups, and data analyzed by paired-group method with determination of Σd^2; the level of significance was obtained from the Table of probability(10).

Results. Table I presents the groups, their age ranges, mean ages, number of animals in each, mean hematocrit values and standard errors of the means.

The hematocrit increased with age; mean

‡ Testosterone propionate was supplied by Ciba Pharmaceutical Products, Inc. Estradiol benzoate by Schering Corp.

§ Water-soluble testosterone, 17-β, diethylaminoethyl-carbonate hydrochloride was synthesized by Ciba Pharmaceutical Products, Inc. and supplied through the generosity of Dr. C. H. Sullivan. An additional amount was generously supplied by Dr. Albert Segaloff of Alton Ochsner Medical Foundation.

in older Group II was significantly higher than in younger Group I ($P<.01$).

Castration depressed the hematocrit; mean of untreated castrates, Group III, was significantly lower than that of intact Group II ($P<.01$). In self-controls, Group IV, the mean after castration was lower than prior to gonadectomy ($P<.01$).

Both androgens restored the hematocrit after castration, the mean of castrates in Group VII, injected with water-soluble testosterone, was significantly higher ($P<.01$) than that of untreated castrates in Group III and not significantly different from that of intact animals in Group II. The mean of Group VI, castrates injected with testosterone propionate in S.O., was not significantly different from that of intact animals in Group II or intact animals given S.O. alone, Group VIII.

The hematocrit of neonatal-castrates, Group XII, was not significantly different from that of intact adults in Group II, and was significantly higher than that of castrates, Group III ($P<.01$).

It is uncertain whether S.O. alone caused an increase in the hematocrit. The mean in intact animals of Group VIII given S.O. is higher than that in untreated Group II ($P<.05$) and the mean of Group V, castrates given S.O., is higher than that of untreated Group III, but not significantly.

Discussion. Within the 50-115 day life span in these experiments, the hematocrit of intact male rats increased with age. These results parallel a reported rise in hemoglobin from 50th to 150th day(1).

The hematocrit response following castration and subsequent androgen therapy confirms previous findings(2-7). The approximate 4-point fall after castration in self-controls is especially significant in light of predictable increases suggested by the results in Groups I and II.

In both intact and castrated groups treated with estradiol benzoate mean hematocrits were lower than corresponding untreated groups, although the differences were statistically non-significant. These results parallel Korenchevsky and Hall's(7), who found that estradiol benzoate-butyrate ester did not alter hematocrits of castrated males. However, it has been reported that estrogen caused a drop in red cell volume(4).

No reference was found regarding hematocrits of adult neonatally castrated rats. Hematocrits of these neonatal castrates, taken at 84-111 days, were not significantly different from those of intact animals and were thus significantly higher than those of animals castrated as adults and examined 14 days later. These results may confirm Korenchevsky and Hall's findings(7) that 100-150 days after castration hematocrits did not differ from those of intact animals. These findings of Korenchevsky and Hall and our own, may indicate secretion of extragonadal androgen.

There was a tendency for both intact and castrated animals injected with S.O. alone to have higher hematocrits than their controls. These results are equivocal but interesting because S.O. is frequently employed as a vehicle for hormones, and because other androgen-like activity of S.O. has recently been reported(11).

Summary and conclusions. 1. Some of our results confirm previous findings: hematocrits of rats castrated as adults are lower than those of intact animals; testosterone propionate returned hematocrits to normal after castration, but did not cause elevation in hematocrits of intact or castrated animals above normal. Estradiol benzoate did not significantly lower hematocrits of intact and castrate rats. 2. Other findings are new: hematocrits of neonatally castrated adult rats were not significantly different from those of intact adults of the same age, and thus differed from those of animals castrated as adults and examined 14 days later. Aqueous testosterone returned the hematocrits of castrates to normal. There was a trend in both intact and castrates for sesame oil to increase the hematocrit. Because sesame oil is widely used as a supposedly neutral vehicle for hormones, the possibility that it has an effect upon the hematocrit warrants further investigation.

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Relation Between P-Aminohippuric Acid Synthesis and Amino Acid Incorporation into Protein.* (23041)

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Systems carrying out synthesis of "peptidic" links, as in formation of hippuric acid, glutamine, glutathione, and pantothenic acid, are considered useful models for the study of peptide formation because of their accessibility and relative simplicity. These reactions have yielded useful information about possible steps leading to peptide bond formation and have provided a basis for a working hypothesis of protein biosynthesis(1). The 4 "model" systems have at least two things in common; namely, carboxyl activation and utilization of energy of one of the pyrophosphate linkages of ATP. It appeared worth while to determine whether a change in endocrine environment affecting peptide synthesis would influence protein formation in a qualitatively similar fashion. To answer this question it was decided to study the "PAH synthetase" system described by Cohen and McGilvery(2-4) while simultaneously studying the rate of protein synthesis. Response of each of these parameters to an evoked change in one of them provides the basis of this report. Measurement of rate of uptake of isotopically labelled amino acids into the protein fraction was chosen as the means of assessing rate of protein synthesis.

Methods. Studies were confined to liver slices from rats in the "hyperthyroid" and "hypothyroid" state. Males of the Sprague-Dawley strain were used. Maintenance on a diet containing 0.5% thyroid powder for 5 weeks was sufficient to produce hyperthyroidism in rats weighing 50-60 g at the outset. Similarly, thyroid function was suppressed significantly by a diet containing 0.05% propylthiouracil fed for 5 weeks. Degree of thyroid dysfunction was gauged by resting metabolic rate (determined as described in 5). From 60-75 mg wet weight of tissue slice was added to each incubation flask, containing 0.001 M PAB and 0.01 M glycine in phosphate-buffered Krebs-Ringer solution. Para-aminohippuric acid (PAH) was determined colorimetrically by the Bratton and Marshall procedure after differential extraction of PAB from PAH according to the method of Cohen and McGilvery(2). PAH synthetase activity is expressed as γ of PAH formed/mg dry weight of tissue/3 hours. In isotope experiments, uptake of glycine-1-C^{14} and lysine-2-C^{14} into the protein fraction of liver slices was determined as follows: Tissue slices (60-75 mg wet weight) were incubated at 38°C for 1 and 2 hours in phosphate-buffered Krebs-Ringer solution containing 0.001 M

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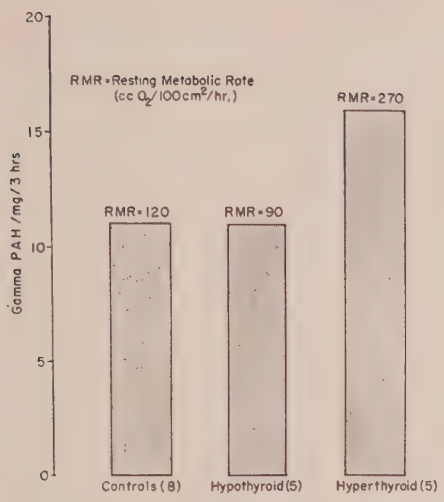

FIG. 1. Synthesis of PAH by rat liver slices. No. in parentheses indicate No. of animals in each group. Details pertaining to individual groups are given in text.

PAH and 0.01 M glycine or lysine. Duplicate flasks were used. The reaction was stopped by addition of trichloroacetic acid (TCA). The slices were then homogenized in 10% TCA solution containing unlabelled glycine or lysine. After centrifuging and washing 4 times with TCA wash, the lipids were removed by extracting twice at 50-55°C with a 3:1 alcohol-ether mixture. The preparations were plated on aluminum planchets and activity determined by counting in a windowless flow counter. Results, corrected to infinite thinness, are expressed as counts/minute/mg protein.

Results. The influence of thyroid hormone on the rate of PAH synthesis is shown in Fig. 1. Slices from control animals synthesized an average of 12 γ of PAH/mg dry weight/3 hours incubation. Propylthiouracil feeding, although depressing metabolic rate significantly, had no influence on rate of synthesis. On the other hand, hyperthyroidism, induced by feeding thyroid powder, not only increased the resting metabolism but provoked a 40% increase in rate of PAH synthesis.

Table I contains data of an experiment testing PAH synthesis simultaneously with rate of incorporation of labelled glycine and lysine into the protein of liver slices from normal and hyperthyroid rats. *Total* radioactivity in each flask containing glycine was approximately 4 million counts/minute in total volume of 4 cc. In the case of lysine, *total* activity (associated with 1-lysine) in each flask was approximately 2 million counts/minute.

It is seen that though hyperthyroidism is associated with an increase in synthetase activity, there is not a corresponding alteration of the degree of amino acid uptake into protein.

Rate of uptake of lysine varied less from slice to slice than did uptake of glycine. After one hour incubation with lysine, average counts/minute/mg of protein were in the narrow range of 16.9 to 18.0 in slices from both normal and hyperthyroid animals.

Protein synthesis (as measured by uptake studies) and formation of the peptidic bond of PAH appear to be dissociated phenomena. This in no way detracts from the usefulness of such model reactions in studying mechanisms involved in peptide synthesis.

Summary. Activity of the enzyme system concerned with synthesis of the peptidic link in p-aminohippuric acid was studied simul-

TABLE I. Incorporation of Labelled Amino Acids into Protein of Liver Slices from Normal and Hyperthyroid Rats.

Animal	Degree of incorporation (avg counts/min./mg protein) Glycine-1-C^{14} Incubation time 1 hr	Glycine-1-C^{14} Incubation time 2 hr	Lysine-2-C^{14} Incubation time 1 hr	Lysine-2-C^{14} Incubation time 2 hr	PAH synthesis/mg dry wt/3 hr	Resting metabolic rate, cc O_2/100 cm²/hr
Normal	63.7	68.0	18.0	24.4	11.3	110
"	61.4	118.1	17.1	23.9	13.7	121
Hyperthyroid	55.6	77.9	16.9	22.9	17.1	266
"	42.0	90.4	17.1	16.2	17.0	326

taneously with rate of uptake of isotopically labelled glycine and lysine into the protein of rat liver slices. The synthetase system increased by 40% in the hyperthyroid state. Incorporation of labelled amino acids, on the other hand, was uninfluenced in the same situation.

Thanks are due Dr. M. P. Schulman for generously supplying the isotope used.

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Restraint Hypothermia and I^{131} Uptake by Rat Thyroid.* (23042)

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Oxygen consumption in rats restrained in the cold is initially higher than in nonrestrained animals at the same environmental temperature. Soon, however, both body temperature and oxygen consumption fall in restrained animals while they remain unchanged in nonrestrained controls(1). Since not all energy expenditure in muscle activity appears as heat, the initially higher oxygen consumption in the restrained, struggling animals need not represent an increased heat production if the basal heat production was so lowered that the total heat production was inadequate for maintaining normothermia. In point of fact, cold and restraint lower liver nonprotein sulfhydryl compounds(2-4) which might indicate some defect in intermediary metabolism and a consequently lowered basal heat production. None of these data, however, clearly distinguish whether the falling metabolism is the cause or the effect of the hypothermia. Since rate of iodine uptake is believed to reflect metabolic activity of the thyroid gland, chief regulator of basal body metabolism, the present experiments on thyroid uptake of I^{131} were undertaken to explore further the effect of restraint in the cold on basal metabolism.

Methods. In studying rate of iodine uptake by the thyroid as affected by restraint and cold, 175-225 g adult male Sprague-Dawley rats were injected at zero hours with 5 μc I^{131} (as KI), immediately divided into 2 groups (one of which was restrained in loose-fitting wire mesh cylinders, flattened on the underside and closed at the ends) and put into a cold room (5° ± 2°C). Then at 1, 2 and 4 hours equal numbers of restrained and nonrestrained animals were killed with ether and their thyroids removed to glass micro slides and air dried overnight. I^{131} activity of the dried glands was determined with a scintillation counter from 10 one-minute (later one 3-minute) counts per gland pair. This was usually done on the day following death, but in any case the measurement was corrected for background (avg 255 cpm) and for decay from zero time. Six animals in each of the 6 groups were used on each of 10 days, making a total of 360 rats. In many of the 180 restrained animals colonic temperature was also recorded at intervals during the 4 hour period. To ascertain the relation between body temperature and thyroid iodine uptake in restrained and nonrestrained animals, thyroid I^{131} activity in 25 of the 4-hour restrained rats (average colonic temperature 17.8°C) was compared with that in 25 extra injected nonrestrained controls in which the

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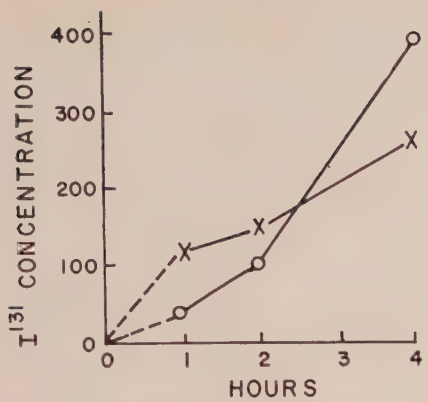

FIG. 1. Thyroid I^{131} concentration (expressed as counts/min.) in restrained (×) and nonrestrained (○) rats at 5° ± 2°C.

body hair had been wetted before 4 hours of cold exposure. This treatment brought their average colonic temperature down to 17.3°C. Thyroid iodine uptake could not be followed *in vivo*, because of the masking effect of I^{131} in blood and tissue fluids adjacent to the thyroid and because the struggling of the animals too frequently shifted the thyroid gland relative to the sensing element. The also potentially desirable technic of determining the rate of release of radioactive thyroxin could not be used in these short term experiments either, because in one hour only 5% of the labelled hormone was lost, an amount comparable to the experimental error and large in comparison with the differences between control and experimental animals.

Results. Fig. 1 gives mean thyroid I^{131} counts, with standard errors including both counter and background variations, for restrained and nonrestrained rats. Total uptake was significantly higher in the restrained animals at one hour ($p<.05$) and lower at 4 hours ($p<.05$). The mean colonic temperature of the restrained rats was 18.2°C at 4 hours.

The thyroid counts for 25 restrained 4-hour rats, of mean colonic temperature 17.8°, averaged 251 ± 25, whereas those in 25 nonrestrained but wetted controls, with mean temperatures of 17.3°, averaged 104 ± 18. The difference was significant ($p<.01$).

Discussion. The difference in I^{131} uptakes might be ascribed to more rapid dissipation of glycogen in the restrained struggling rat as compared to the control, with the subsequent fall in temperature and metabolic rate being due to diminished reserves. This agrees with the observation that restrained rats which struggle more become the more hypothermic (5). It is not in agreement, however, with the data showing greater thyroid I^{131} uptake in restrained (struggling) rats than in equally hypothermic nonrestrained (quiet) controls.

The significantly higher thyroid I^{131} count of restrained rats, at the one-hour measurement, as compared to the control animals, reflects a much greater rate of accumulation of I^{131} (Fig. 1). This early increased rate of I^{131} accumulation in the restrained rat, however, gives way to a lesser rate of uptake as compared to the control, for at the 4-hour comparison the control thyroid I^{131} count was significantly greater than for the restrained. These changes correlate well with the rate of oxygen uptake in the cold where restrained rats initially have a greater rate, and after approximately 1½ hours, a progressively lesser rate than nonrestrained controls(1). Although the response of the thyroid is usually regarded as being too slow to account for an increased O_2 consumption occurring in one hour or less, strong presumptive evidence favoring such a hypothesis is the observation by Booker *et al.*(6) that 1½ hours of cold exposure to both normal and adrenalectomized mice results in a 100% increase in the oxygen consumption of live homogenate.

The parallel falls in body temperature, rate of thyroid iodine uptake and rate of O_2 consumption in restrained rats exposed to between 2 and 4 hours of cold give no clue as to whether falling heat production causes the hypothermia or the reverse. The fact that both the rate of I^{131} uptake and O_2 uptake are above corresponding control values at one hour is not conclusive either, for both these may have been caused by the greater struggling of the restrained rats. However, the fact that thyroid iodine uptake was much higher in restrained rats 4 hours in the cold than in nonrestrained but equally hypothermic controls indicates that restraint causes hypothermia by some mechanism other than

depression of heat production. The drop in body temperature of the restrained rat, in fact, appears to be the cause of the drop in oxygen consumption (metabolism), for if the reverse were true the I^{131} uptake (thyroid metabolism) should have been higher in the nonrestrained than in the restrained animals.

Summary. Thyroid I^{131} uptake by restrained rats was higher at one hour, and lower at 4 hours, than that of nonrestrained control animals. The lower the body temperature of the restrained animals at 4 hours, the lower the I^{131} uptake by the thyroid. Restrained hypothermic rats had a significantly greater thyroid uptake of I^{131} than equally hypothermic nonrestrained animals. It is concluded that hypothermia of restrained animals can not be ascribed to depressed basal metabolism.

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Association of Mouse Pathogenic Strain of Echo Virus Type 9 with Aseptic Meningitis.* (23043)

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Echo viruses are a new group of agents which have been isolated from stools of aseptic meningitis cases and of normal children(1). Although one of the 14 antigenic types, echo-6, has repeatedly caused aseptic meningitis(2-5), the etiological relationship of most types to human disease remains obscure. The present paper offers evidence that echo-9 virus has caused one epidemic of typical aseptic meningitis near Cambridge, England, during 1955, and two epidemics of this syndrome with milder symptoms in other nearby areas during 1956.

Methods. Virus isolations and antibody studies were performed in cultures of human amnion cells(6) which had been grown for the first 3 days in medium containing 20% human serum and 0.5% lactalbumin hydrolysate in Gey's balanced salt solution followed by 3 days in medium containing 10% inactivated rabbit serum and 0.5% lactalbumin hydrolysate in balanced salt solution. Immediately prior to inoculation, the cell sheets were washed twice in salt solution and a maintenance medium, containing 5% rabbit serum and 0.25% lactalbumin hydrolysate in salt solution, was added. Uninoculated cultures remained intact in maintenance medium for 10 days. Monkey kidney (MK) cell cultures were also used. Those in Cambridge were kindly provided by Dr. J. O'H. Tobin, while those used in New Haven were prepared by methods described elsewhere(7). Virus isolations were attempted from feces obtained from patients between 2 and 7 days after onset of illness. In the third outbreak, virus isolation was attempted from both throat washings and rectal swabs. Samples of serum were collected from each patient between 2 and 7 days and again between 12 and 21 days after onset of illness. Neutralization tests on paired sera were performed on serial

* The laboratory work carried out in New Haven was aided by a grant from Natl. Fn. for Infantile Paralysis.

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TABLE I. Clinical Features of Eleven Cases of Aseptic Meningitis in 1955.

Clinical features	No. of cases
Symptoms and signs	
Fever	11
Stiff neck	10
Headache	10
Vomiting	8
Backache	8
Sore throat	3
Limb pains	2
Photophobia	2
Paralysis	0
CSF findings (2 to 10 days after onset)	
<10 cells	1*
10- 24 "	2
25- 99 "	2
100-300 "	2
>300 "	4
> 10 "	10

* This patient had no cells in the CSF 2 days after onset, but on seventh day the CSF contained 282 cells/mm^3.

fivefold dilutions of sera using 300 to 1000 TCD_{50} of virus in amnion cultures and 320 TCD_{50} in MK cultures. Virus typing was carried out in MK cultures using antisera against each of the 14 echo types, against the cytopathogenic Coxsackie types, and against the 3 polioviruses.

Results. Bourn outbreak. Between Oct. 17 and Nov. 11, 1955, 8 cases of typical aseptic meningitis occurred amongst members of 4 families who lived in an emergency housing area at Bourn, Cambridgeshire. Three additional cases occurred in people living in Cambridge who were in close association with the first 2 Bourn cases. The incubation period was between 8 and 11 days. The clinical features are listed in Table I. All at some stage had increased lymphocytes in the cerebrospinal fluid (CSF), ranging between 10 and 1180/mm^3, and 8 cases also had between 6 and 48 granulocytes/mm^3. In the case of L.R., the CSF contained no cells on second day of illness but on the seventh day it contained 261 lymphocytes and 21 granulocytes mm^3.

Virus was isolated in amnion cultures from feces obtained from two patients, L.M. and L.R., on second and third days of illness, respectively. Virus was also isolated from L.R.'s feces by inoculation of MK cultures. At the Virus Reference Laboratory, Colindale, virus was isolated from the feces of 2 more patients, E.K. and C.W. (G.P.B. Boissard, personal communication). These 4 virus strains could not be distinguished from each other on the basis of neutralization tests using 1000 TCD_{50} of virus against the acute and convalescent sera from patient L.R. (Table II).

Virus from patient L.R. (Bourn virus) in its first passage in amnion culture had a titre of $10^{7.9}$ TCD_{50}/ml when titrated in cultures of human amnion. This material was used in most neutralization tests. After 6 passages in MK cultures, the titre increased to $10^{8.5}$/ml. Bourn virus was not neutralized by antisera against the Coxsackie viruses or polioviruses. In repeat tests it was neutralized by antisera against echo-9 virus but not by antisera against any of the other 13 types of echo virus (see Table III). Both Bourn and Quigley strains produced irregular-shaped plaques with diffuse boundaries on MK cells under agar, typical of group A echo viruses (8). Paired sera of 2 patients were also titrated in MK cultures against the Quigley strain of echo-9 virus. The results are comparable, as shown for patient L.M. in Table III, with those obtained when the same sera were tested against the Bourn strain.

TABLE II. Serological Relationships of Viruses Isolated from Cases of Aseptic Meningitis. (Each virus was tested against sera of the patient yielding the virus and also against the paired sera of patient L.R.)

Date of epidemic	Patient	Days after onset	Serum titres against 1000 TCD_{50} of virus isolated from each patient: L.R.'s sera	Homologous sera
Oct. 1955	L.R.	3	0*	0
			50†	50
"	L.M.	2	0	0
			>100	>100
"	E.K.	6	0	50
			250	>250
"	C.W.	7	0	50
			>50	50

This legend pertains for Tables III through VI, also.

* Titre of acute serum. 0 indicates < 10.

† Titre of convalescent serum.

TABLE III. Cross Reactions between Echo-9 Sera and the Bourn Virus.

Virus	Serum titres (50% endpoint against 100 TCD_{50} of virus) Echo-9, Hill strain*	Echo-9, Quigley strain*	Bourn patient L.M. Acute	Conval.	Other†
Hill‡	15,625	1,500			<10
Quigley§	15,625	2,500	0	250	<10
Bourn	5,380	220	0	>100	<10

* The echo-9 typing sera were prepared by immunization of monkeys, the Hill serum by Wenner and the Quigley serum in New Haven.

† Includes echo viruses 1-8, 10-14, Coxsackie A9, B1-5, polio 1-3.

‡ Hill strain, the echo-9 prototype, was isolated from a normal child in Ohio(10).

§ Quigley strain was isolated from a patient with aseptic meningitis, in W. Va.

Acute and convalescent sera were collected from each patient. Sera were also collected from 14 family contacts 23 days after the epidemic began, and 9 of these were bled again on the 43rd day. Neutralization tests which were performed against 1000 TCD_{50} of Bourn virus showed that 5 cases and 2 contacts had no antibody in the first serum but acquired antibody by the time the second serum was taken. Two cases with a low antibody titre in the first serum showed fivefold or greater rise in antibody titre in the second serum, and 4 cases and 5 contacts had a high antibody titre in the first serum with no increase in the second serum (Table IV). In addition, 3 contacts had antibody in single specimens of serum taken 3 weeks after the epidemic began, one contact had a low antibody titre in both serum samples, one contact had no antibody in either early or late serum, and 2 contacts had no antibody in single serum samples. Thus, of a total population of 23 at risk, 17 subjects were infected with echo-9 virus and 11 of these became ill with aseptic meningitis.

Huntingdon outbreak. During late July and early Aug. 1956, many servicemen and their families who lived on Royal Air Force Station near Huntingdon suffered from severe headache and fever and in some cases this was accompanied by a morbilliform rash. No patient had neck stiffness, and lumbar puncture was not performed. In 3 families who were

TABLE IV. Neutralization Tests on Sera Taken during the Bourn Epidemic, October, 1955.

Name	Virus isolation	Days after onset	Antibody titre against echo-9 virus
		Cases	
M.B.	n.t.	7	>250
		20	"
R.C.	0	4	50
		16	>250
M.M.	0	5	"
		20	"
I.M.	0	3	50
		16	250
C.W.	+*	6	"
		18	"
E.K.	+*	5	0
		17	50
G.R.	0	5	0
		18	250
L.R.	+	3	0
		16	50
L.M.	+	2	0
		13	250
H.D.M.	0	3	0
		13	>250
R.K.	0	5	"
		14	"
		Contacts	
B.M.		23†	0
		43	>50
H.Z.B.		24	0
		49	0
Ro.C.		23	>100
		43	"
E.A.C.		23	10
		43	"
P.L.		23	>100
		43	"
D.L.		23	"
		43	"
E.R.R.		23	"
		43	"
J.R.		23	"
		43	"
P.R.		23	0
		49	>50
B.R.		21	0
A.R.		21	10
A.C.		27	>50
L.C.M.		23	0
B.B.		24	50

* Virus isolated by Dr. G. P. B. Boissard.

† Echo-9 virus isolated from a rectal swab taken on this date.

n.t. = not tested.

TABLE V. Neutralization Tests on Sera Taken during the Huntingdon Epidemic.

Virus strain	Sera of patients: J.C.	M.R.	J.E.R.	D.R.	L.M. (Bourn)
Patient J.C.	10	20	0	n.t.	10
	>50	>250	>250	n.t.	>250
Patient M.R.	n.t.	0	0	0	0
	>250	>250	>250	>250	>250
Bourn strain	n.t.	0	0	n.t.	0
(Echo-9)	>250	>250	50	n.t.	>250

studied closely, stool specimens and paired sera were obtained from 2 patients, stool and convalescent serum was obtained from one patient, stool only was obtained from another patient, and from two further patients paired sera were obtained. The families lived some distance apart from each other. Echo-9 virus was isolated from 3 stools. Rising titres of neutralizing antibody to 2 current strains of virus and the Bourn strain of echo-9 virus were detected in all 4 serum pairs and a high titre was detected in the single convalescent serum. The results are shown in Table V.

Cambridge outbreak. During late Sept., 1956, a schoolboy, D.W., developed severe headache and fever but there was no neck stiffness and no rash. During the next 3 days, 3 of his studymates complained of the same symptoms. All had played a trumpet belonging to D.W. on the day prior to onset of D.W.'s illness, which was the date on which they resumed school after the summer vacation. Throat washings, rectal swabs, and sera were collected from each patient 3 days after D.W. became ill; further serum samples were obtained 16 days later. Echo-9 virus was isolated from throat washings obtained from J.A.C. A rising titre of neutralizing antibody against 300 TCD_{50} of this strain of virus was detected in paired sera from all 4 cases (Table VI).

Mouse pathogenicity. Newborn mice were inoculated with the 3 strains of echo-9 virus, Hill, Quigley, and Bourn, using undiluted tissue culture fluids containing 10^7, $10^{6.5}$, and $10^{8.5}$ TCD_{50} of virus, respectively. From 51 to 90 mice were inoculated with each strain, some injected intracerebrally and some subcutaneously. None of the mice inoculated with the Hill or Quigley strain succumbed to the virus, in confirmation of the earlier findings with these strains. However, the mice inoculated with the Bourn strain developed paralytic illness, with all 19 animals injected subcutaneously succumbing on the third and fourth days and 27 of 32 mice injected intracerebrally coming down between the third and sixth days. The virus was carried for 3 passages in mice. Eviscerated torsos of paralyzed mice yielded titers of more than $10^{8.5}$ and of $10^{7.2}$ per gram, respectively, in MK cultures and in newborn mice. Echo-9 (Hill) antiserum neutralized, both in TC and in mouse neutralization tests, large doses of Bourn virus harvested from infected mouse torsos; thus, a serum dilution of 125 neutralized 10^6 TCD_{50} of the second passage mouse virus in MK cultures, and over 10^4 paralytogenic doses of the same virus seed in the mouse neutralization test(9). Sections of paralyzed mice manifested myositis indistinguishable from that produced by group A Coxsackie viruses. No lesions were seen in the brain or fat pads.

TABLE VI. Neutralization Tests on Sera Taken during the Cambridge Epidemic.

Virus strain	Sera of patients: J.A.C.	C.W.C.	J.H.R.	D.A.W.
Patient J.A.C.	0	0	0	10
	250	>50	>50	50

Discussion. Echo-9 strains have been recovered from healthy children(1,10) and also from sporadic cases of aseptic meningitis(1).‡ However, the present study is the first association of this virus with a sharp epidemic of frank aseptic meningitis and subsequently with two outbreaks characterized by milder symptoms. Since echo-9 virus was the only virus isolated from the patients studied, it seems reasonable to infer that this virus caused each outbreak. Rising titres of neutralizing antibody to current strains of echo-9 in paired sera from patients further strengthen this view. From these results it would appear that echo-9 virus, as well as echo-6(2-5), must be added to the list of agents which may cause the aseptic meningitis syndrome. Sup-

‡ Unpublished results.

port of this view has recently come from several sources. A virus isolated in MK cells by Dr. P. DeSomer in Louvain from the spinal fluid of an aseptic meningitis patient, and sent to New Haven for typing, turned out to be echo-9 virus. DeSomer§ isolated the same virus from the spinal fluids of 7 cases of aseptic meningitis and from 26 fecal specimens. Serological studies showed eightfold or greater antibody increases during convalescence. Tyrrell and Snell(11) in England have recently described an epidemic disease characterized by rash and aseptic meningitis, some cases of which were similar to the disease observed in babies in East London in 1954 by Crawford, *et al.*(12). Echo viruses were isolated in tissue culture by both groups, but only strains isolated in 1955 and 1956 were found to produce myositis and paralysis in infant mice. Similar viruses, isolated from cerebrospinal fluids, throat washings, and feces, have been recovered during the summer and fall of 1956 from other outbreaks in different parts of England(13). We have learned through personal communications from these investigators that the viruses isolated in their studies have now been typed and have turned out to be strains of echo-9 virus. It is noteworthy that several virus strains, recovered in tissue culture by Meyer§ from a large outbreak of aseptic meningitis which occurred in Germany during the fall of 1956, were also typed as echo-9 virus.

The observation that certain strains of echo-9 virus produce myositis and paralysis in infant mice raises the question of whether the echo-9 strains really belong with the echo or with the Coxsackie viruses. In addition to the mouse pathogenicity of certain echo-9 strains, the Quigley strain of this virus, isolated from an aseptic meningitis patient in West Virginia in 1954, has produced mild lesions in the spinal cord of monkeys inoculated directly into the lumbar area.‡ The lesions, including cellular infiltration and neuronophagia, were limited to the lumbar area of the spinal cord, but were present outside the area of traumatic injury produced by the injection. Benyesh recovered virus from the spinal cords of a number of monkeys for at least a week after inoculation.

Summary. From an outbreak of 11 cases of aseptic meningitis near Cambridge, England, during the autumn of 1955, echo-9 virus was isolated from 4 patients. In addition, 7 cases and two family contacts showed rising titres of neutralizing antibody to this virus. From 2 subsequent outbreaks of febrile headache in this region during the summer and autumn of 1956, echo-9 virus was isolated from 6 patients and rising titres of neutralizing antibody were detected in sera from 8 patients. These data, plus the recent echo-9 isolations from spinal fluids as well as from the alimentary tract of aseptic meningitis cases in other outbreaks, strongly suggest that echo-9 virus has caused severe and mild cases of this syndrome. English strains isolated in 1955 and 1956 were found to produce myositis and paralysis in infant mice indistinguishable from that produced by group A Coxsackie viruses.

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Differentiation of "Normal" and Neoplastic Cells Maintained in Tissue Culture by Implantation into Normal Hamsters.* (23044)

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It is generally accepted that cell lines derived from normal tissue and those derived from neoplastic tissue are difficult to distinguish from one another after maintenance in tissue culture(1,2). Experiments were therefore designed to ascertain if biological differences between such cell lines could be demonstrated by application of newer methods of tissue culture(3-8) and transplantation of tissue culture cell lines to a heterologous host(9). Delineation of specific amino acid and vitamin requirements of a mouse fibroblast(3) and a human carcinoma cell line(4) has permitted parallel *in vitro* cultivation of several additional human and animal cell lines deriving from normal and neoplastic tissue(5-8) in media embodying these same minimal requirements supplemented with 10% whole serum. Studies with these cell lines *in vitro* have failed thus far to reveal significant biochemical or morphological differences between those derived from normal and from neoplastic tissue. Similarly, studies on susceptibility of cell lines originating from normal *vs.* malignant tissue to certain carcinolytic agents(10) resulted in the paradoxical observation that either was equally susceptible to a given agent, despite the fact that these same compounds exhibit selective anti-tumor activity in experimental animals. Observations during the past 18 months on quantitative titration of tissue culture cell lines in the cheek pouches of golden hamsters(9) suggested that all cell lines do not behave similarly in this heterologous host. The purpose of this report is to record the results of experiments which indicate that despite the aforementioned *in vitro* similarities, there are significant differences in the biological behavior of different cell lines maintained *in vitro*, and that these differences may correlate with the source of the culture.

Methods. The cell lines studied are listed in Table I. These strains were maintained in media prepared as described by Eagle(3-5) in which the essential serum protein was provided by 10% whole, pooled, human serum, except for 4 animal cell lines, where serum protein was provided by 10% whole horse serum. All of these cell lines exhibit remarkably similar rates of growth in these media, and with the exception of Sarcoma 180 and L-929, which are of fibroblast-like morphology, resemble one another in morphologic appearance. They are best described as "epithelial-like" in the sense that individual cells are predominantly polygonal, with distinct, intensely basophilic nuclei, and their characteristic growth in essentially monocellular layers directly on glass surfaces gives the appearance of "pavement" epithelium. The layer of cells growing on the surface of culture flasks was removed for animal experiments by replacing the culture fluid in 5-7-day-old cultures with fresh media containing Difco "1:250" trypsin in final concentration of 0.125% and incubating the flask *circa* 5 min at 37°C. The cells so dispersed were sedimented at 5-600 rpm, washed 2-3 times with fresh medium to remove the trypsin and redispersed with gentle agitation in appropriate volumes of media. After enumeration by haemocytometer counts, the suspension of cells was diluted so that the desired inoculum was contained in a volume of 0.1 ml of medium. Syrian hamsters, 60-80 g in wt were prepared under light nembutal anaesthesia and the desired inoculum implanted under the epithelium of the cheek pouch with a 24-gauge needle. Both cheek pouches of 6 hamsters were implanted with each inoculum of each cell line and in each instance, 3 of 6 hamsters were treated with

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TABLE I. Titration of Tissue Culture Inocula in Unconditioned Hamsters.

Cell line	Tissue of origin	*In vitro* age (mo)†	Development of tumors* following implantation of cells at: 1×10^6	1×10^5	1×10^4
Sarcoma 180	Biopsy, S-180 in CFW mice (Foley)	18	+	+	+
KB‡	Biopsy, human, epidermoid carcinoma (Eagle)	23	+	+	+
HeLa‡	" " cervical " (Gey)	69.5	+	+	+
J-111‡	Peripheral blood, human, acute monocytic leukemia (Osgood)	27	+	+	+
Wilms'-6	Biopsy, human, Wilms' tumor (Foley)	4.5	+	+	+
Amnion	Human amnion at term (Foley)	3.5	+	+	0
407‡	" embryonic intestine (Henle)	23	+	+	0
LLC-M-1§	Hypertrophic lymph node, mouse of unknown strain (Hull)	61	+	0	0
L‡	Biopsy, human, liver (Chang)	31	+	0	0
Thymus	" " thymus (Foley)	5.5	+	0	0
Endometrium	Normal, human, therapeutic abortion (Foley)	5.5	+	0	0
Lung	Biopsy, human, lung‖ (Foley)	5.5	+	0	0
L-929	Connective tissue, C3H mouse (Earle)	194	+	0	0
Kidney¶	Normal Rhesus monkey	.5	+	0	0

* See text.
† To Dec. 1, 1956.
‡ Strains obtained through courtesy of Dr. Harry Eagle, N.I.H., Bethesda, Md.
§ *Idem*, Dr. R. N. Hull, Eli Lilly Research Labs., Indianapolis, Ind.
‖ Chronic pneumonitis.
¶ Primary *in vitro* cultures. Trypsin-dispersed suspensions of 1×10^5 and 1×10^4 cells implanted directly also failed to grow in unconditioned hamsters.

cortisone acetate in subcutaneous doses of 2-3 mg, administered simultaneously at time of implantation and twice weekly post-implantation. *The 3 remaining hamsters in each instance were not treated with cortisone.* At least 2 such experiments were done with each cell line, thus the data summarized in Tables I and II represent observations on *circa* 800 hamsters. Cheek pouches of all animals were observed twice-weekly under light nembutal anaesthesia for 60 days. Development of a nodule which increased progressively in size and became vascularized within a few days was considered as evidence of growth. All cell lines recorded as positive in Table I produced such tumors in two or more cheek pouches per experiment in more than one animal. These tumors may regress spontaneously in unconditioned hamsters, but are readily transplanted to other hamsters within the first 10-14 days. Histological sections were prepared from all cheek pouches in which such tumors developed. The behavior of cell lines recorded as negative in Table I was distinctly different. In most instances, the original implant disappeared completely within 24 hrs, but occasionally persisted as a tiny nodule which regressed spontaneously within 5-6 days. In no instance did the original implant develop progressively or become vascularized, and they were not transplantable to other hamsters. Histologically, such nodules consist of a necrotic focus at site of implantation, surrounded by the usual inflammatory response.

TABLE II. Titration of Tissue Culture Inocula in Hamsters Conditioned with Cortisone Acetate.

Cell line†	Development of tumors* following implantation of cells at: 1×10^5	1×10^4	1×10^3	1×10^2	10
S-180	+	+	+	+	+
KB	+	+	+	+	+
HeLa	+	+	+	+	+
407	+	+	+	0	0
L	+	+	0	0	0

* See text. † See Table I.

Results. All cell lines studied, irrespective of tissue of origin, grew prolifically in cheek pouches of hamsters conditioned with cortisone acetate when the inoculum contained 1 x 10^5 or more cells. However, *as summarized in Table I, this was not the case with unconditioned hamsters.* Although each cell line so studied grew when 1 x 10^6 cells were implanted in cheek pouches of unconditioned

hamsters, results with inocula containing 1 x 10^5 and 1 x 10^4 cells clearly indicate that all strains which can be maintained *in vitro* do not exhibit identical *in vivo* biological properties. Thus far, the failure of 1 x 10^5 cells in most instances, and the uniform failure of 1 x 10^4 cells to grow in unconditioned hamsters appears to be characteristic of those cell lines deriving from non-malignant sources. That this failure to grow in unconditioned hamsters is not entirely due to length of time the cell line has been in tissue culture is evident from Table I, since long-established (LLC-M-1, L, L-929) as well as more recently isolated (Thymus, Endometrium, Lung) cell lines failed to grow under these experimental conditions.

In sharp contrast, all cell lines deriving from neoplastic tissue produced characteristic tumors in 33-100% of cheek pouches of unconditioned hamsters implanted with 1 x 10^5 or 1 x 10^4 cells, and a number of tumors so induced have been maintained through several transplants in unconditioned hamsters. The uniform ability of such cell lines to grow under these experimental conditions also is noteworthy, since it is conceivable that the tumor from which these cells were isolated may have contained normal stromal cells. Again, this ability to grow in unconditioned hamsters is not simply a reflection of length of time the cell line has been maintained *in vitro,* as evidenced by comparison of *in vitro* ages of strains KB, HeLa, and J-111 with those of S-180 and Wilms' 6.

Experiments in which tissue culture inocula were titrated further in cheek pouches of hamsters conditioned with cortisone acetate give further evidence of differences in biological behavior of cell lines derived from different sources. Thus far (Table II) cell lines isolated from neoplastic tissue produce tumors following implantation of as few as *circa* 10 cells, whereas cell lines derived from adult non-malignant tissue fail to produce tumors, even in conditioned hamsters, when inocula of 1 x 10^3 or fewer cells are used. The strain of normal embryonic cells so studied (407, Table II) exhibits a growth potential intermediate between that of cell lines derived from malignant and adult non-malignant tissue. Further studies on the titration of these and additional cell lines, including a number isolated from aspirated bone marrow of patients with and without neoplasm(6), in conditioned and unconditioned hamsters are in progress.

Discussion. The factors of adaptation and/or selection determining successful isolation of mammalian cells *in vitro* have not yet been fully delineated. Cell lines exhibiting different growth potentials have been isolated from cultures derived from malignant(11), and non-malignant sources(12-14). Thus, it is of considerable interest that morphologically similar tissue culture cell lines exhibit distinctly different biological properties when exposed to physiological influences of an intact heterologous host. Differences in behavior in unconditioned hamster cheek pouches of cell lines used in studies on cytotoxic activity of carcinolytic agents in tissue culture(10) suggest that despite the similarity in response to such agents *in vitro,* significant biological differences between these cell lines indeed exist, and that further *in vitro* study of such cell lines may reveal biochemical attributes related to these differences in growth potential.

It is perhaps more than coincidence that cell lines derived from neoplastic tissue exhibit a greater growth potential than those derived from non-malignant sources when titrated in unconditioned hamsters. Moore *et al.*(15) mentioned that cell lines derived from normal sources failed to grow in unconditioned rats, while previous experience in these laboratories with neoplastic or embryonic and heterologous adult normal tissue implanted directly into the hamster cheek pouch by trocar or as trypsin-dispersed cell suspensions indicates that under these conditions, adult normal tissue fails to grow. These observations are in accord with Greene's suggestion(16) of heterotransplantability as a criterion for distinguishing between neoplastic (or embryonic) and adult normal tissue. The present studies, however, must be extended to additional cell lines and the results correlated with behavior of tissue cells under other biological conditions both *in vivo* and *in vitro* before

differences in growth potential exhibited in the unconditioned hamster can be evaluated as differential evidence of malignancy in general. However, the present data can be interpreted as representing distinct biological differences among those cell lines derived from normal and neoplastic tissues which can be maintained in tissue culture.

Summary. Quantitated inocula of 14 tissue culture cell lines which are remarkably similar *in vitro* were implanted into cheek pouches of unconditioned Syrian hamsters. All cell lines grew in the cheek pouch when 1 x 10^6 cells were implanted, but only those cell lines derived from neoplastic tissue produced tumors when inocula contained 1 x 10^4 cells. Other experiments indicated that this difference in the growth potential of cell lines deriving from neoplastic tissue also may be delineated in hamsters conditioned with cortisone acetate by implantation with 1 x 10^3 or fewer cells.

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Antagonism of Insulin-Induced Gastrointestinal Hypermotility in the Rat.* (23045)

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Antispasmodic potencies of many drugs have been expressed in terms of relative degree of antipropulsive action shown in the Macht and Barba-Gose charcoal meal test(1). The results of this test are informative, but effects of drugs on normal propulsive motility may not reflect useful effects expected in treatment of gastrointestinal malfunctions associated with smooth muscle spasm and hypermotility. Some clinically useful agents, such as adiphenine hydrochloride (Trasentine) and dicyclomine hydrochloride (Bentyl), which exert their smooth muscle inhibiting effect in "musculotropic" or "papaverine-like" fashion, are without significant inhibitory effect in the charcoal meal test except in doses which produce pronounced central nervous system effects. Atropine sulfate and methantheline bromide (Banthine) require subcutaneous doses of 2 mg/kg and 15 mg/kg respectively to produce a 50% decrease in normal propulsion. Such high doses of these agents might be expected to be responsible for extraneous effects which could be antagonistic or synergistic with peripheral antipropulsive action of the compound. The charcoal meal test alone fails to indicate ex-

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TABLE I. Charcoal Meal Test. Effect of drugs on normal propulsive motility of stomach and small intestine of rat.

No. of rats	Agent	Dose, mg/kg	Route of admin.	Avg length intestine traversed (% ± S.D.) † 20 min.	40 min.
25	Saline		S.C.		61.5 ± 12.0
20	"		Oral	51.4 ± 9.5	
5	Atropine SO_4	1	S.C.		50.8 ± 10.3
10	*Idem*	2	"		34.3 ± 6.1
10	"	4	"		26.7 ± 5.4
10	"	8	"		35.7 ± 5.1
5	"	16	"		36.1 ± 5.3
5	"	32	"		33.0 ± 13.6
5	"	8	"	27.6 ± 11.0	
5	"	2	Oral	30.1 ± 3.5	
5	"	8	"	36.9 ± 9.8	
5	"	16	"	37.1 ± 3.5	
5	Methantheline br.	2	S.C.		56.1 ± 8.2
5	*Idem*	8	"		52.2 ± 6.9
15	"	16	"		39.0 ± 3.9
10	"	32	"		13.1 ± 5.0
5	"	100	Oral	24.2 ± 6.1	
10	Scopolamine methylbr.	1	S.C.		31.0 ± 4.3
10	*Idem*	2	"		28.5 ± 6.5
5	Papaverine HCl	100	"		54.9 ± 10.6
10	*Idem*	200*	"		21.7 ± 10.7
10	"	400*	"		6.6 ± 6.7
5	"	200*	Oral	54.5 ± 9.2	
5	"	400*	"	32.0 ± 6.3	
10	Dicyclomine HCl	125	S.C.		38.8 ± 5.5
5	*Idem*	250*	"		21.3 ± 5.4
10	Adiphenine HCl	200*	"		53.2 ± 13.2
10	Hexamethonium br.	2	"		53.6 ± 11.2
10	*Idem*	4	"		41.0 ± 9.5
10	"	8	"		31.9 ± 8.2
5	"	16	"		37.1 ± 10.6

* Symptoms of depression. † Distance to farthest point of charcoal.

tent to which acetylcholine blocking capacity, ganglionic blocking capacity, or direct smooth muscle inhibiting capacity of a compound contributes to its total antipropulsive action. Additional use of a similar *in vivo* test, which would allow comparison of effect of the drug on both normal and hyperactive states of intestinal smooth muscle of the same species, should help answer this question.

Methods. Adult rats of the Mead Johnson colony (McCollum strain) and of either sex weighing between 200 and 400 g were caged without food and water for 24 hours prior to experiment. The test drug was injected subcutaneously and followed immediately by subcutaneous dose of 40 units/kg of insulin (Squibb), each in 2 ml/kg volume, and each injected at a different site. Thirty minutes later 5 ml/kg of the charcoal mixture (6% charcoal and 1% methocel 400 in water) was administered orally. Forty minutes after administration of the charcoal mix the animals were sacrificed with diethyl ether and stomach and small intestine removed. The distance from the stomach to the nearest area which contained the first one cm, or more, of charcoal and also the distance from stomach to farthest point containing one cm or more of charcoal, were expressed as % of total length of small intestine. The insulin-treated rats were somewhat physically depressed but in experiments on 800 rats, only one convulsion was seen during the 40-minute waiting period. Distribution of the charcoal mixture in insulin-treated rats is different from that in the Macht and Barba-Gose rats and to our

knowledge the distribution in insulin-treated animals has never been described. Forty to 50 minutes after oral administration of 5 ml/kg of charcoal mixture, according to the procedure of Macht and Barba-Gose, this mixture is rather uniformly distributed in the proximal 60% of small intestine and the stomach appears to be rather full. In the insulin-treated rats the stomach is small and firm with relatively little retained material. No charcoal mix is found in the proximal 34% of the intestine (average of 60 rats), and the farthest point containing one cm or more of the charcoal is usually 70 to 80% of length of small intestine. The major, and most consistent, result of insulin administration was the "cleaning out" of stomach and proximal 34% of small intestine. This result is apparently in agreement with the conception that the predominant effect of insulin is exerted, via autonomic nerves, from hypoglycemic stimulation of vagal centers in the central nervous system and that the stomach and more proximal portions of the gastrointestinal tract are more richly supplied with vagal fibers(2). It was found that there was an approximately linear relationship between the log-dose of antipropulsive agents and the average inhibition of insulin effect as determined by measuring the distances from stomach to beginning of charcoal mix. Although insulin caused an increase in the distance traversed by the farthest portion of the charcoal mix, this increase was not well defined, and it soon became apparent that there may be no relationship between the effect of a drug on inhibiting the "cleaning out" action and reducing the distance of the farthest point of travel. This latter measurement was therefore not used in determining ability of drugs to antagonize the hyperpropulsive effects of insulin. The procedure employed in the charcoal meal test was the same except that rats received no insulin, and the farthest point at which one cm or more of the charcoal mix was found was used to calculate the % intestine traversed. The difference in this distance between treated rats and control rats was expressed as % inhibition. Effectiveness of the following antispasmodic agents was compared in the 2 tests: atropine sulfate, papaverine hydrochloride, methantheline bromide (Banthine), adiphenine hydrochloride (Trasentine), dicyclomine hydrochloride (Bentyl), scopolamine methylbromide (Pamine), and hexamethonium bromide. All doses were based on weight of the salt.

TABLE II. Effect of Drugs on Gastrointestinal Hyperpropulsion Induced by Subcut. Admin. of Insulin in the Rat.

No. of rats	Agent	Dose, mg/kg	Route of admin.	Avg distance,* stomach to charc'l mix at 40 min., % ± S.D.
60	None			34.4 ± 8.2
10	Atropine SO_4	.1	S.C.	21.2 ±11.0
5	*Idem*	.25	"	18.9 ± 7.2
10	"	.5	"	6.6 ± 4.4
10	"	1	Oral	27.5 ± 5.1
20	"	2	"	15.3 ±16.4
10	"	4	"	4.7 ± 4.8
10	Methantheline	.5	S.C.	19.4 ± 7.2
10	br.	2	"	7.9 ± 6.5
5	*Idem*	4	"	5.5 ± 4.3
10	"	40	Oral	20.9 ±10.3
15	"	80	"	7.5 ± 5.0
10	Scopolamine	.0125	S.C.	24.7 ± 5.3
10	methylbr.	.05	"	15.2 ± 4.2
10	*Idem*	.15	"	0
10	"	4	Oral	20.0 ±13.7
10	"	8	"	15.2 ± 4.6
10	"	12.5	"	2.6 ± 4.2
10	Papaverine HCl	12.5	S.C.	18.3 ± 6.5
10	*Idem*	25	"	14.8 ± 6.7
10	"	50	"	2.8 ± 0.3
10	†Adiphenine HCl	50	"	19.4 ±14.1
5	*Idem*	100	"	18.1 ± 2.6
10	Dicyclomine	5	"	22.0 ± 4.6
10	HCl	10	"	20.0 ± 9.1
10	*Idem*	20	"	14.8 ± 6.8
10	Hexametho-	1	"	24.4 ± 8.6
10	nium br.	2	"	16.2 ± 5.4
5	*Idem*	4	"	0

* Distance to nearest point of charcoal.
† Commercially available solution containing glucose.

Results. Table I and Table II list the results obtained in the charcoal meal test and insulin test. Since some investigators have administered the compounds orally and utilized a 20-minute waiting period in their charcoal meal tests, some data obtained in this manner are included for purposes of comparison.

The relative potency of each drug in both

TABLE III. Comparison of Potency of Several Antipropulsive Agents as Measured by Different Methods in the Intact Rat.

Drug	Charcoal meal test S.C. ED_{50}*	Insulin antipropulsion test (mg/kg)	Ratio, char. meal ED_{50}/insulin ED_{50}
Atropine SO_4	2.4	.146	16.4
Methantheline br.	16.5	.66	25.0
Scopolamine methylbr.	1.05	.028	37.5
Papaverine HCl	165	15.5	10.6
Dicyclomine HCl	157	13.0	12.1
Hexamethonium br.	8†	1.82	4.61

* ED_{50} was determined from log dose-response relationships and represents approximate dose required to produce an avg of 50% inhibition of propulsion as described in methods.

† Produced an avg of 48% inhibition in group of 10 rats.

the charcoal meal test and insulin antipropulsion test is shown in Table III. To eliminate variability of absorption from the gut, all comparisons were made from data obtained with subcutaneous injections of antipropulsive agents.

The slopes of the log dose-response lines for atropine, methantheline and scopolamine methylbromide in the insulin antipropulsive test were very similar, while those of dicyclomine and papaverine were much flatter but similar to each other. With the exception of scopolamine methylbromide and hexamethonium bromide, antipropulsive potencies, relative to atropine, were quite similar in either test. Papaverine and dicyclomine, when compared to atropine, were more effective in the charcoal meal test and relatively less effective in the insulin test, whereas the reverse was true for methantheline and especially scopolamine methylbromide. The results indicate that the more a compound relies upon its papaverine-like effects to inhibit normal propulsion, the nearer to unity will be the ratio between the ED_{50} in the two tests.

The data summarized in Table IV give some indication of the specificity and limitations of the insulin antipropulsion test. The results are in agreement with the general concepts of the actions of these drugs except in the case of dibenamine and phentolamine. Despite the fact that the known peripheral effects of these agents would not be expected to interfere with the insulin-induced hypermotility, it is seen that they are capable of producing pronounced inhibition in doses incapable of affecting normal propulsion. The possibilities of parasympathetic ganglionic blocking action or a central blocking action are now being investigated.

Discussion. Methantheline is known to possess approximately ½ the ganglionic blocking capacity of hexamethonium(3), and since it was only half as potent as hexamethonium in the charcoal meal test, its ganglionic action was probably of importance. In the insulin antipropulsion test, on the other hand, methantheline was 3 times as potent as hexamethonium, making available only ⅙ the necessary ganglionic blocking activity required to reduce insulin-induced propulsion. The fact that hexamethonium was so efficient in abolishing hyperpropulsive action of insulin is consistent with the idea that extrinsic nerve impulses affect the increased motility of the gut. The inhibitory effect exerted by procaine and lidocaine is probably due to ability of these agents to interfere with ganglionic transmission. The comparative potency of various ganglionic blocking agents will be the subject of a subsequent report.

TABLE IV. Effect of Subcutaneous Injections of Various Agents on Normal Propulsion and Insulin-Induced Hyperpropulsion in the Intact Rat.

No. of rats	Agent	Dose, mg/kg	% inhibition: Charcoal meal test	% inhibition: Insulin antipropulsion test
10	Hexamethonium	1		29.1
10	br.	2	12.9	53.0
5	*Idem*	4	33.4	100.0
10	"	8	48.2	
5	"	16	40.0	
5	Pyrilamine maleate (Neoantergan)	2	0	0
10	Phenoxybenzamine	.1		39.1
5	(Dibenzyline)	1	0	100.0
5	Phenoxybenzamine	10	41.7	
10	Phentolamine HCl	.2		28.8
10	(Regitine)	2	0	72.8
10	*Idem*	10	16.6	
10	Procaine HCl	6		31.3
5	*Idem*	40	23.0	
10	Lidocaine HCl	2		46.6
5	(Xylocaine)	10	4.0	

Atropine appears to be definitely limited in its ability to inhibit normal propulsion as shown by the fact that a subcutaneous dose of 2 mg/kg is apparently as effective as 16 times that dose. The results suggest that higher doses of atropine introduce a factor which opposes peripheral inhibition of propulsion. An exaggeration of this phenomenon could be expected in the case of new synthetic agents whose actions may be less specific than those of atropine and other agents included in this report.

The insulin antipropulsion test has several advantages over the charcoal meal test for comparing activity of new antipropulsive agents. Besides economy and convenience afforded by the fact that smaller doses of drugs are required, one is much less likely to encounter unwanted side effects which may prevent a true evaluation of the antipropulsive potency. Because papaverine-like agents, in otherwise asymptomatic doses, are capable of producing pronounced inhibition of the propulsive effect of insulin, one is much less likely to overlook an important agent of this type. Finally, a comparison of the effect of the compound in both tests may be very informative.

Summary. A new intestinal antipropulsive test in intact insulin-treated rat is described. The insulin test provides certain advantages over the commonly-used charcoal meal test for screening of synthetic antispasmodic or antipropulsive agents. The use of both tests permits determination of the effect of the agent on both normal and hyperactive states of intestinal muscle and provides information relative to the mechanism of action of the agent. The specificity and some limitations of the test are discussed.

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Glomerular Filtration Rate and Renal Plasma Flow in Cholera and Acute Gastroenteritis. (23046)

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Cholera is characterized by acute purging and vomiting leading to marked dehydration of body and acute depletion of plasma volume. The renal plasma flow is likely to be interfered with and either transitory or permanent damage to kidney might be possible. Death is mostly due to development of anuria which indicates failure of function of the kidney. Hence assessment of kidney functions is of importance to predict prognosis, to learn progress of the disease and to suggest treatment. Anuria is invariably present but not so in conditions of acute gastroenteritis, though clinical features resemble each other closely. Whether the hemodynamic factors in the formation of urine are altered in the above 2 conditions, either due to effect of specific toxins or to depletion of plasma volume, remains to be elucidated. In the present investigation glomerular filtration rate and renal plasma flow were determined in patients suffering from cholera and acute gastroenteritis. The same studies were also carried out in normal persons for comparison.

Methods. Selection of cases. All patients suffering from cholera and gastroenteritis admitted into the Nilratan Sircar Medical College Hospitals, with clinical features of acute purging, vomiting and showing signs of dehydration were selected. As soon as patients were admitted, their dehydration was corrected by transfusion of hypertonic saline followed by normal saline with sodium bicarbonate or hypertonic glucose(25%) with calcium gluconate and ascorbic acid. Patients whose stool on culture did not show *vibrio*

cholerae were taken to be cases suffering from acute gastroenteritis. When dehydration was controlled, there was improvement in clinical condition and normal urination was established 2 to 13 days from onset of the disease, glomerular filtration rate and renal plasma flow were determined by inulin and para-amino hippuric acid clearance tests respectively. Patients admitted into the surgical ward with fracture of long bones, hernia, hydrocele, gun shot injury of limbs, etc., when recovered and fit to be discharged were selected as normal subjects for determination of inulin and para-amino hippuric acid (PAHA) clearance tests. These patients came from the same strata of society as those suffering from cholera and acute gastroenteritis. *Inulin and PAHA clearance tests.* For measurement of glomerular filtration rate, inulin and for measurement of renal plasma flow PAHA, were used as test substances. Subjects drank 40 oz water, one hour before the test, in the morning after breakfast. A sterile rubber catheter was passed and retained to drain the urinary bladder continuously. The bladder was evacuated continuously and a portion of urine kept to be used as blank. 5 cc blood was withdrawn from the anticubital vein and through the same needle 30 cc freshly prepared 10% inulin in normal saline mixed with 4 cc 20% PAHA dissolved in normal sodium hydroxide were introduced, and the time when the injection was completed was noted. The needle was then fitted to a transfusion set and 250 cc of .9% sodium chloride containing 35 cc of 10% inulin and 10 cc of 20% PAHA, were introduced at 4 cc/minute. At intervals of 30, 50 and 90 minutes after injection, 5 cc blood samples were withdrawn from the anticubital vein of the other arm. Urine samples which accumulated during each experimental period were also separately collected and every time the bladder was washed with 20 cc saline followed by injection of air. The washings were mixed with urine and total volume of urine collected during each experimental period noted. After collection of final sample of blood and urine, the catheter and injection needle were removed. Blood samples were collected in vials containing a mixture of ammonium and potassium oxalate crystals. Some subjects had shivering during the transfusion. Inulin was estimated by the method of Roe(1) and PAHA by the method of Bratton and Marshall(2). From total amount of inulin and PAHA excreted in the urine samples, collected between 30 and 50 minutes and between 50 and 70 minutes after injection of these solutions, amounts of these substances excreted in urine/minute were calculated. From plasma concentration of inulin and PAHA, volume of blood plasma cleared of these substances in urine in one minute was calculated. The results are given in Tables I and II.

TABLE I. Inulin and PAHA Clearance Values in Normal Subjects and in Patients Suffering from Cholera.

Subjects	Inulin clearance (glomerular filtration rate), cc/min.	PAHA clearance (renal plasma flow), cc/min.
Cholera (14)	60 ± 12*	272 ± 46
Normal (19)	114 ± 8	517 ± 46
Diff. of means	54	245
Stand. error of diff.	14.07	62.44
t	3.8	3.9

* Mean ± stand. error.
Figures in parentheses indicate No. of subjects.

TABLE II. Inulin and PAHA Clearance Values in 6 Patients Suffering from Acute Gastroenteritis.

Inulin clearance, cc/min.	PAHA clearance, cc/min.
41 ± 16	193 ± 64

Results. Inulin clearance (glomerular filtration rate) was 114 cc/minute in normal subjects, 60 cc in cholera patients and 41 cc in patients suffering from acute gastroenteritis. PAHA clearance (renal plasma flow) was 517 cc/minute in normal subjects, 272 in cholera patients and 193 cc in patients suffering from acute gastroenteritis.

Discussion. In patients suffering from cholera and acute gastroenteritis both the values for glomerular filtration rate and renal plasma flow were diminished and the differences were statistically significant. From postmortem studies on kidney of patients who died of shock, Bywaters and Dible(3) reported pallor of cortex and congestion of

medulla. They could not account for such changes in the kidney. Trueta *et al.*(4) ascribed this interference of blood flow to persistent arterial spasm demonstrated experimentally to accompany shock. Tomb(5) was of the opinion that anuria and renal damage in shock were due to anoxia of the kidney. De and Sen Gupta(6) demonstrated evidence of cortico-medullary diversion of blood flow in the kidneys, in shock associated with various surgical and obstetrical conditions and in cholera. All patients suffering from cholera, who were investigated, survived and became normal. This excluded any possibility of permanent parenchymatous lesion in kidneys. Therefore in conditions like cholera where acute purging and vomiting results in circulatory deficiency, the cortico-medullary diversion of blood flow might take place. The diminished circulatory blood volume in acute stage of cholera might produce anoxia resulting in renal cortical vasospasm and diversion of blood flow through the medulla. The low figures obtained in clearance values are the result of diminished blood flow. This state of circulation persists for a number of days, even after correction of circulatory deficiency. If renal anoxia is of short duration, so as not to cause permanent damage to the kidney, normal circulation is gradually established with the onset of diuresis. It has, however, been shown that no change in oxygen content of whole blood of cholera patients takes place(7).

The clearance values obtained in patients suffering from acute gastroenteritis were similar to those of cholera cases. These observations are likely to exclude the possibility of any specific toxin acting on the kidney.

Summary. Glomerular filtration rate and renal plasma flow were determined by inulin and para-amino hippuric acid clearance tests in 19 normal subjects, in 14 cholera patients and in 6 patients suffering from acute gastroenteritis. In patients suffering from cholera and acute gastroenteritis, glomerular filtration rate and renal plasma flow were greatly diminished to the same degree, when these patients had apparently normal urination after transfusion of saline. The diminished clearance values might be due to renal anoxia causing temporary diminution in blood flow through the cortex of the kidney and not due to any specific toxins of the disease.

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Antagonistic Effect of Thiocarbanilide on 4,4′-Dinitrocarbanilide, A Component of Nicarbazin. (23047)

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During the course of studies on compounds related to nicarbazin, a complex of 4, 4′-dinitrocarbanilide and 2-hydroxy-4, 6-dimethylpyrimidine, it was observed that most of these compounds were ineffective as anticoccidial agents(1). Thiocarbanilide and several of its derivatives were among the ineffective compounds. Since structural analogues may competitively antagonize the pharmacological effects of certain drugs(2), we explored several compounds structurally similar to 4, 4′-dinitrocarbanilide (DNC), the active component in nicarbazin, for antagonistic effect on this anticoccidial agent. The results presented in this paper show that thiocarbanilide will competitively antagonize the anticoccidial and egg shell depigmenting(3) effects of nicarbazin.

Materials and methods. The compounds studied were supplied by Dr. L. H. Sarett and Dr. R. C. O'Neill.* The standardized methods described by Cuckler, Malanga and Ott(4) were used for evaluating anticoccidial activity of combinations of compounds. Analyses for DNC(5) were made on plasma samples from chicks on day 8 and plasma and yolk samples from hens on days 10-12. The depigmenting effect of nicarbazin on egg shell color was determined by the following method. A series of 8 shells, each graded in 0.5 units and varying in shade of color from chalk white (0) to deep brown (4) were maintained as index for color scoring. A new set of shells was prepared weekly. Shell scores for each hen were obtained during 7 to 10-day control period and during experimental period of 10-12 days. Color scores of the first 3 days of production during the experimental period were excluded from data to eliminate the period of sub-maximal drug effect on shell pigmentation.

Results. The following compounds, structurally related to 4, 4′-dinitrocarbanilide, were examined for antagonistic effect on the anticoccidial activity of nicarbazin: 4, 4′-dinitrocarbanilide; 2, 2′-dinitrocarbanilide; N, N′-diallyl-4, 4′-dinitrocarbanilide; N, N′-di(4-nitrophenyl)-p-nitrobenzamide; 4, 4′-dinitrobenzanilide; 4, 4′-dinitroazobenzene; 4, 4′-dichlorocarbanilide; 4, 4′-difluorocarbanilide; 4, 4′-disulfamylcarbanilide; thiourea; sulfathiourea; phenylthiourea; thiocarbanilide; 3, 3′-dichlorothiocarbanilide; and 4, 4′-dichlorothiocarbanilide. The last 3 compounds listed inhibited the anticoccidial effect of nicarbazin in a 1 to 1 ratio or less, whereas all other compounds were ineffective. Thiocarbanilide (sym. diphenylthiourea) was selected for further studies.

As shown in Table I, 1 part of thiocarbanilide to 4 of nicarbazin in the feed of chickens partially antagonized the protective effect of nicarbazin on mortality resulting from cecal coccidiosis. Higher concentrations of thiocarbanilide in the feed completely antagonized the anticoccidial effect of nicarbazin. The antagonistic effect of thiocarbanilide was also shown by the increased number of oocysts produced in chicks fed mixtures of thiocarbanilide and nicarbazin in ratios of 1 to 4 or less.

Thiocarbanilide may be administered parenterally and still exert its antagonistic effect on nicarbazin. For example, the data showed that thiocarbanilide, when administered in daily subcutaneous dosage of 12 mg/kg of body weight, completely inhibited the protective effect of 0.015% nicarbazin on mortality from cecal coccidiosis (Table II). A daily subcutaneous dosage of 50 mg of thiocarbanilide/kg body weight was required to antagonize the anticoccidial effect of 0.03% nicarbazin in the feed. When the amount of nicarbazin was further increased to 0.06% in the feed, thiocarbanilide in a subcutaneous

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TABLE I. Effect of Graded Feed Concentrations of Thiocarbanilide on Therapeutic Activity and Plasma Levels in Chicks Fed 0.015% Nicarbazin.

% thiocarbanilide in feed	% coccidiosis mortality‡		Millions of oocysts produced/chick		DNC, γ/cc plasma	
	Cont.*	NCB†	Cont.	NCB	Cont.	NCB
.0	35	0	33	<0.1	0	1.41
.0045		0		"		1.12
.0009		0		"		1.33
.0019		0		"		1.22
.0038		15		31		.68
.0075		40		29		.58
.015		15		59		.43
.030	40	60	29	62	0	

* Cont. = Non-medicated feed. † NCB = 0.015% nicarbazin in feed ‡ 20 chicks/group.

dosage of 100 mg/kg of body weight had only slight antagonistic effect. Furthermore, when thiocarbanilide was administered subcutaneously, a daily dosage of 6 mg/kg of body weight completely antagonized the suppressive effect of 0.015% nicarbazin on oocyst formation. Larger dosages of thiocarbanilide were required to antagonize the oocyst suppressing effect of 0.03 and 0.06% nicarbazin in the feed.

The antagonistic effect of thiocarbanilide for nicarbazin appears correlated with blood concentrations of DNC obtained in the chicks. When chicks were fed 0.015% nicarbazin, the plasma concentration of DNC was about 1.5 γ/ml (Tables I, II). However, by the subcutaneous administration of thiocarbanilide in a dosage of 100 mg/kg, the concentration of DNC in blood may be decreased to a nondetectable level. Likewise, simultaneous feeding of ration containing thiocarbanilide and nicarbazin in a 1 to 1 ratio reduced the concentration of DNC in the blood to about 25% of that obtained with nicarbazin alone.

Further evidence of the antagonistic effect of thiocarbanilide for nicarbazin was obtained when feeds containing both compounds in various ratios were fed to laying hens. When nicarbazin alone was fed to heavy breed hens, which normally lay brown shell eggs, the shells had considerably less pigmentation. However, when nicarbazin and thiocarbanilide were fed simultaneously in a 1 to 1 ratio, the depigmenting effect of nicarbazin was completely nullified (Table III). When feed mixtures containing thiocarbanilide and nicarbazin in ratios of 1 to 2 were fed, the depigmenting effect of nicarbazin on egg shells was only partially antagonized. Plasma levels of DNC in hens fed nicarbazin and thiocarbanilide in a 1 to 1 ratio were considerably less than in hens fed only nicarbazin (Table III). Also, hens fed rations containing both nicarbazin and thiocarbanilide produced eggs with less DNC in the yolk than in those from hens fed nicarbazin alone. Presumably, these de-

TABLE II. Effect of Subcutaneous Dosages of Thiocarbanilide on the Therapeutic Activity and Plasma Levels in Chicks Fed 0.015% Nicarbazin.

Subcut. dose thiocarbanilide, mg/kg/day*	% coccidiosis mortality‡		Millions of oocysts produced/chick		DNC, γ/ml plasma	
	Cont.†	NCB†	Cont.	NCB	Cont.	NCB
0	18	0	28	<0.1	0	1.61
1.5	30	0	20	1		1.48
3	30	0	21	1		1.01
6	30	0	25	24		.80
12	0	40	16	12		.34
25	0	20	19	12		.19
50	20	30	6	13		.13
100	60	90	8	41		.0

* Admin. daily throughout experiment. † Cont. = Non-medicated feed; NCB = 0.015% nicarbazin in feed. ‡ 20 chicks/group.

TABLE III. Effect of Thiocarbanilide on Egg Shell Color-Score and Plasma and Yolk Concentrations of DNC during Feeding of Nicarbazin.

% nicarbazin in feed	% thiocarbanilide in feed	Shell color score period Cont.	Exp.	DNC conc. Plasma, γ/ml	Yolk, γ/g
.008	None	2.9	.5	2.45	9.34
	.002	2.5	1.7	.99	11.10
	.004	3.2	2.8	1.16	5.70
	.008	2.7	2.8	.96	5.07
.0120	None	3.2	.5		20.55
	.003	2.8	.2	1.98	21.20
	.006	3.7	1.9	1.54	15.50
	.0120	2.1	2.1	.78	9.52
	.020	2.9	2.8	.33	1.25
.0125	None	3.5	.5	2.02	20.4
	.0125	3.5	3.5		
	.025	2.9	2.3	.52	1.85

creased plasma and yolk DNC levels reflected a decreased assimilation of nicarbazin by the laying hen because of the thiocarbanilide in the feed.

Discussion. The anticoccidial and egg shell depigmenting effects of nicarbazin are related to the amount of DNC absorbed from the intestinal tract. Plasma levels of DNC are correlated with both of these effects, which in turn are proportional to concentration of nicarbazin in feed(4,6). Thiocarbanilide effectively antagonized the anticoccidial activity and shell depigmenting effect of the structurally related component (DNC) of nicarbazin. The antagonism occurred either by feeding or injecting thiocarbanilide and simultaneously feeding nicarbazin. Thus, the decreased plasma and yolk DNC concentrations caused by thiocarbanilide appear to be due to an interference with nicarbazin absorption.

Summary. 1) Thiocarbanilide, in a 1 to 1 ratio or less, effectively inhibited the anticoccidial activity of nicarbazin when given parenterally as well as orally. 2) Thiocarbanilide also antagonized the effect of nicarbazin on the depigmentation of egg shell color. 3) In both instances, the antagonistic effect of thiocarbanilide was correlated with decreased plasma concentrations of dinitrocarbanilide. 4) It is suggested that thiocarbanilide may interfere with the activity of nicarbazin through competition in absorption.

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Influence of Beta-Aminopropionitrile upon Development of Connective Tissue in Croton Oil Pouches. (23048)

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Injection of air and dilute croton oil into subcutaneous tissues of rats produces a well-defined pouch(1). The wall of the pouch after 6 days is composed principally of fibroblasts and minimal numbers of blood vessels. When beta-aminopropionitrile (BAPN) is fed to rats daily in drinking water, the development of a croton oil pouch is retarded. Because BAPN retards development of croton oil pouches, it seemed desirable to examine gross and microscopic findings in these pouches, and to analyze some important constituents of connective tissue to resolve whether there are also differences in concentration of such components. The 2 components selected for analyses were hexosamine and hydroxyproline. These constituents were chosen because hexosamines are an im-

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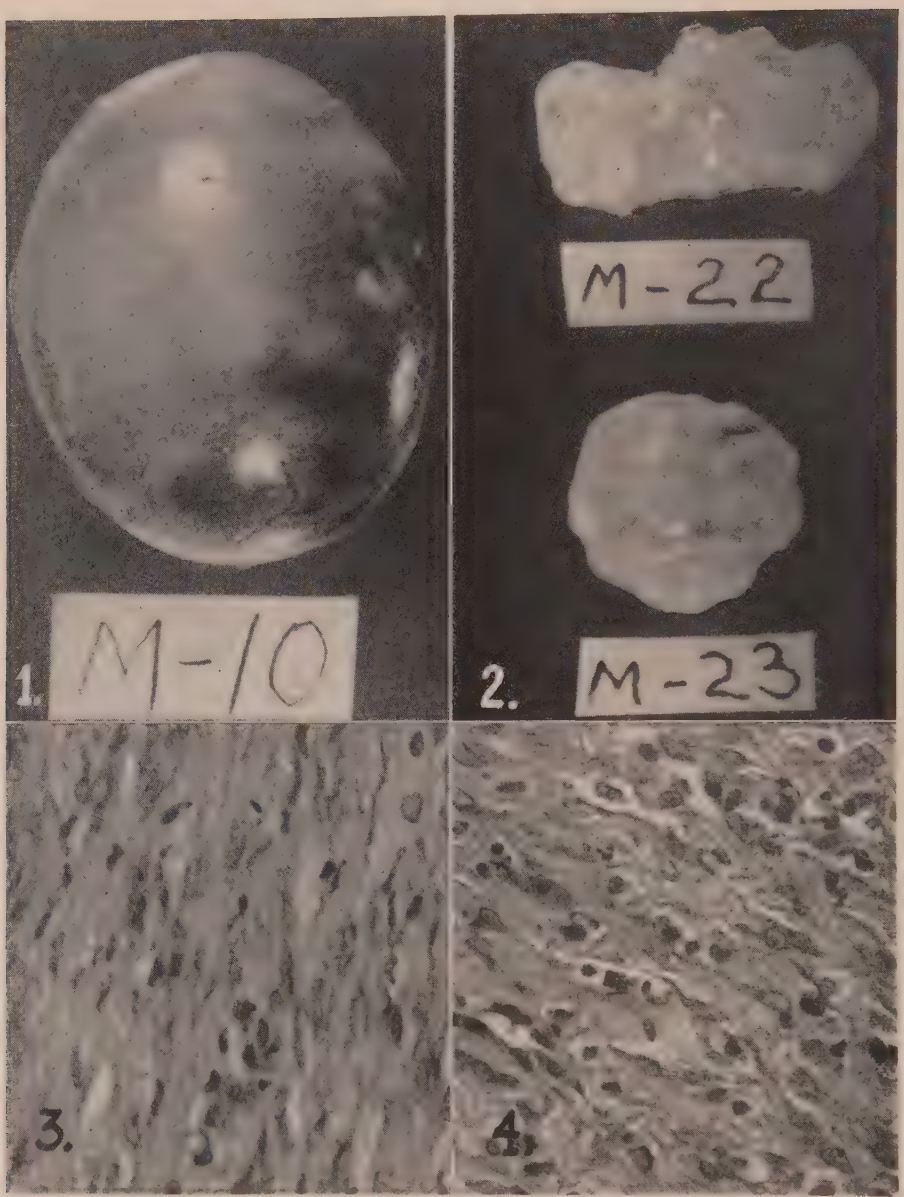

FIG. 1. Eighteen day croton oil pouch from control rat approximately actual size. Note relatively smooth exterior and well developed capsule.

FIG. 2. Two 18 day pouches from test animals are collapsed, smaller and their surfaces irregular.

FIG. 3. Section of capsule from control pouch stained with hematoxylin and eosin. Fibroblasts tend to align along one plane. Many nuclei are fusiform and collagen is abundant. × 350.

FIG. 4. Immature fibroblasts with round nuclei are numerous and collagen synthesis is diminished. × 350.

portant component of ground substance(2); whereas, hydroxyproline is indicative of concentration of collagen(3).

Method. Rats were fed a commercial diet.† Each day, to every 100 ml of drinking water, 0.2 ml of concentrated alkaline BAPN kept

† Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

at 4°C was added, and this was offered to 18 rats with croton oil pouches. Three control and 3 test rats were killed after 6, 12, and 18 days. The pouches were carefully dissected from surrounding areolar tissue, care taken to exclude visible blood vessels. The inner surface of each pouch was scraped to remove fibrin and inflammatory cells. After several washings in cold (2-4°C) physiologic saline, the pouches were weighed and sections made for microscopy. Thereafter, the pouches were cut into 2-4 mm pieces and placed into 5 ml of cold 4% perchloric acid. At this stage, pouch walls with areas of hemorrhage or necrosis were discarded. The tissues were frozen in liquid air and pulverized in a mortar. The pulverized protein was then subjected to extraction in perchloric acid, ethanol, and ether as recommended by Muller and Herranen(4). Total volume of perchloric acid washings was recorded. Filtered aliquots of perchloric acid washings were subjected to micro Kjeldahl digestion, and total acid soluble nitrogen calculated. Lipids were extracted from the protein by repeated ethanol and ether extractions. Total dry, fat free protein residues were weighed in each case. Ten mg of protein was hydrolyzed in 1 ml of 4 N HCl acid for 15 hours at 100°C. These acid hydrolysates were used for determination of hexosamine by the Boas method(5) and hydroxyproline concentration by the method of Neuman and Logan(3). Aliquots of acid hydrolysate were also subjected to micro Kjeldahl digestion to determine per cent of N in the protein residue.

Results. The results are shown in Tables I and II.

Gross. The test pouches were poorly formed, ill-defined from surrounding areolar connective tissue, and many were collapsed (Fig. 1). They were smaller, both in size and in wet and dry weight as compared to corresponding control pouches. Comparable quantities of oil were obtained in collapsed and control pouches. The pouch from only 1 of 9 rats (No. 16) was comparable in weight to those of control rats (Table II).

Microscopic. The microscopic findings are summarized in Table I. The presence of an

TABLE I. Microscopic Observation of Croton Oil Pouches from Normal and Test Rats Fed BAPN.

Rat No.	Fibroblastic maturation	Schiff positive material	Organization
	Control, Day 6		
1	2+	2+	2+
2	2+	3+	+
3	3+	2+	+
	Day 12		
4	4+	+	2-+
5	3+	+	2+
6	3+	2+	3+
	Day 18		
8	4+	+	4+
9	4+	+	4+
12	4+	+	4+
	Test, Day 6		
13	+	4+	+
14	+	3+	+
15	+	3+	+
	Day 12		
16	2+	3+	+
17	2+	3+	2+
18 & 24*			
	Day 18		
25†	+	4+	2+
29†	2+	4+	+
33†	+	4+	+

* Tissue used for chemical study only.
† From a second series of rats which were given 0.15 instead of 0.2 ml/100 ml of drinking water.

increase in number of fibroblasts with large spherical nuclei associated with less collagen fibers in the stroma per unit area is interpreted as a delay in the rate of maturation of fibrocytes. Such alterations were more pronounced in test pouches. An attempt was also made to evaluate the degree of fibroblastic maturation (1-4+) at 6, 12 and 18 days depending upon the abundance of collagen and the presence of immature (spherical) or mature (fusiform) nuclei. The organization or alignment of fibroblasts and collagen fibers parallel to the surface of the pouch wall was also investigated. Interference with alignment of collagen fibers, and fibroblasts with respect to the surface was more pronounced in the test pouches (Fig. 2 and 3). An approximation of fibroblastic orientation in relation to time was graded from 1-4+ and tabulated. In addition to alterations in the quantity of collagen and cellular variations larger foci of homogeneous eosinophilic material were present in test pouches. Dupli-

TABLE II. Hexosamine and Hydroxyproline Concentrations in Croton Oil Pouches from Normal and Test Rats Fed BAPN.

Rat No.	Wet wt of pouch, mg	Wt of extracted protein, mg	N. in extracted protein, %	Acid extractable N., % total N.	Hexosamine in extracted protein, %	Hydroxyproline in extracted protein, %
			Control, Day 6			
1	1810	138.6	14.2	8.36	1.02	2.70
2	1120	68.9	13.7	9.28	.96	2.96
3	1130	106.2	13.7	10.00	.93	2.97
			Day 12			
4	1720	174.8	14.3		1.03	3.98
5	1700	159.4	14.7	4.1	.88	3.80
6	1740	149.6	13.9	3.4	.94	3.53
			Day 18			
8	1410	124.2	12.9	4.95	.92	4.26
9	1320	119.9	13.2	4.52	.98	4.78
12	1340	97.1	13.7	6.82	.98	4.16
			Test, Day 6			
13	540	44.6	14.6	13.6	.90	1.89
14	650	33.9	13.3	19.5	.93	1.65
15	1000	57.4	13.1	20.1	1.00	1.65
			Day 12			
16	1450	122.5	14.3	11.6	.92	3.66
17	670	37.8	14.4	18.8	1.06	2.48
18 & 24*	810	62.5	14.0	14.9	1.17	2.94
			Day 18			
25†	580	42.5	13.5		1.18	1.88
29†	530	26.8	14.1		.82	3.52
33†	810	35.9	14.1		.98	3.52

* Pouches from 2 rats were combined.

† From a second series of rats which were given 0.15 instead of 0.2 ml/100 ml of drinking water.

cate sections were stained with periodic-acid Schiff in each case(6). Examination disclosed that Schiff positive material which is localized in the homogeneous foci is more abundant in the test pouches.

Chemical. Weights of pouches before and following extraction as well as hydroxyproline and hexosamine concentrations are shown in Table II. Cold perchloric acid extraction will dissolve salts, nucleotides and amino acids. Acid extraction might also dissolve some collagen leaving insoluble collagen for analysis(7). More acid soluble nitrogenous material was obtained from the test than from control pouches. Whether this difference is due to extraction of nucleic acids from the immature fibroblasts or the presence of greater quantities of an acid soluble protein matrix in the stroma of the pouch wall is not known. Nitrogen in the fat free protein residue varied from 12.9 to 14.4%. It is not possible to make any correlation between variations in % N and age of pouch. The hexosamine concentration varied from 0.92 to 1.02 in control pouches. A variation of this degree is probably inherent in the method of hexosamine determination. Although amount of hexosamine in test pouches varied somewhat more widely, the values appear comparable to those observed in controls.

The hydroxyproline concentration is expressed as per cent of protein residue because we wished to investigate variations in hydroxyproline with respect to protein concentration. As one would anticipate, the hydroxyproline concentrations are lowest in 6-day pouches and gradually increase in concentration with time in the control group. In addition to reductions in wet weight and extracted protein residue, there is also less hydroxyproline in the dry, fat free protein from

the test pouches. Examination of hydroxyproline concentration shows that the levels are less in test pouches at 6, 12, and 18 days. Whether hydroxyproline is expressed in per cent of protein residue or in terms of per cent protein N, the concentrations are lower in test pouches. Less hydroxyproline is evident in 8 of 9 test rats. This decrease in concentration of hydroxyproline in test pouches is in agreement with our microscopic observations of delayed collagen synthesis.

Discussion. Feeding BAPN in drinking water results in smaller pouches when compared to those observed in control rats. In addition to gross and microscopic observations, it is also possible to demonstrate less hydroxyproline in the extracted protein from test pouches. Abundant quantities of homogeneous eosinophilic material between immature fibroblasts might either be ground substance or a collagen precursor. The fact that hexosamine concentrations were not significantly increased in the extracted test pouches suggests that the eosinophilic material either is not ground substance or that the mucopolysaccharides were dissolved during perchloric acid extraction. The hexosamine concentrations of extracted protein residue from the pouch are approximately double the values reported by Boas for areolar tissue in young rats(5). Increased hexosamine concentrations in croton oil pouch may be related to the presence of immature and metabolically active fibroblasts.

Microscopic observation of abundant homogeneous eosinophilic material with reduced numbers of collagen fibrils, recoveries of proportionately less fat free protein and lower concentrations of hydroxyproline in the extracted protein from test pouches, all suggest that BAPN in some way exerts an influence on collagen synthesis. The observation of increased solubility in perchloric acid of pulverized protein from test pouches also suggests that they differ from normal. Since the acid extractable nitrogenous material was not analyzed for hydroxyproline it is not possible to say whether there is more acid soluble collagen in the test pouches.

The fibroplasia which is stimulated by croton oil is sufficient to permit chemical analysis with the added advantage that the approximate age of fibroblasts is known. Uncorrected collagen of the skin represents 67.8% of dry fat free weight when calculated from the hydroxyproline content(3). In our studies the hydroxyproline concentrations varied from 1.65 to 4.78% of extracted protein. These concentrations when expressed in collagen equivalents indicate that collagen varied from 14.1 to 35.6% of extracted protein. Robertson and Schwartz have reported delayed collagen and hydroxyproline synthesis in scorbutic guinea pigs injected with extracts of Irish moss(8). In their experiments the delayed synthesis of collagen is related to deficiency of vit. C. BAPN probably effects collagen synthesis in some other way because the rats in our study did not develop any signs of vit. C deficiency.

Summary. Pouches from control and test rats fed BAPN were removed after 6, 12, and 18 days. Extraneous material such as collections of leukocytes, fibrin, and areas of hemorrhage were removed before the pouches were extracted in 4% perchloric acid, ethanol, and ether. Grossly the test pouches were poorly formed, and microscopically they showed decrease in fibroblastic maturation and collagenic fiber formation with relative increase in homogeneous eosinophilic Schiff positive material. Fat free protein residue was analyzed for hexosamine and hydroxyproline concentration. More nitrogenous material was consistently obtained from test pouches during perchloric acid extraction. Hexosamine concentration in different pouches varied from 0.82 to 1.17% of dry fat free protein. Significant alterations with age or variations from control values were not observed. In control pouches the hydroxyproline concentration gradually increased to approximately 4% in 18 days. Hydroxyproline concentrations in the test pouches were significantly less than in controls of corresponding age.

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A Simple, Inexpensive, Continuously-Variable Infusion Pump.* (23049)

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The instrument to be described was developed to fill the need for an infusion pump with which rate of delivery could be continuously varied over a wide range during injection. The present pump has met this need and has been employed for 9 months in experiments on small animals in which rate of injection has been critical.

Details of construction and mode of operation of this pump will be apparent from Fig. 1 and 2. The motor (Bodine Electric Co., Chicago, 1/50 H.P., 1725 rpm, with gear reduction to 11.5 rpm) turns the first drive cone (A). The 4 identical drive cones are knurled cast aluminum. The conical portion is 4″ long and the large and small diameters are 3″ and .95″ respectively. Cone A drives cone B by means of rubber ring (¼″ by 18″ circumference) which provides contact between the 2 cones. The point of contact of the ring with the cones is adjustable by means of yoke K, rod L and setscrew M (Fig. 1). It can be seen that when the largest portion of cone A is driving B, B rotates 3.16 times as rapidly as A; conversely, when the smallest portion of A is driving B, A rotates at 3.16 times the speed of B. Thus, a 10-fold range of speed is achieved by the first pair of cones. Cone C is coupled to B and both turn at the same speed. Cone D, however, is driven by C through a mechanism identical to that linking cones A and B. Range of speed for the 2 pairs of drive cones is therefore 100-fold. To prevent slippage of the cones on the rubber rings it is necessary to have considerable tension on the 2 pairs of drive cones. This is achieved by means of the tension frame (N to P) which is clearly shown in Fig. 2. This frame carries cones B and C on supports N and N′ and holds them under tension against cones A and D by means of the 2 springs on rod O. The springs are turned in opposite directions, and tension on them can be varied by means of nut and setscrew P.

Cone D turns the threaded ½″ shaft F through the sprocket and chain drive E (Fig. 1). The syringe plunger is driven by rider G which is carried along as shaft F turns. This rider is made of two brass bars ½″ x 1″ x 4″ which are clamped together and drilled and threaded to receive shaft F. The 2 halves of the rider are then hinged at one end and closed with a pivoted pin at the other end so that they can be opened and moved on F. Also on this rider is bar I which can be set to contact switch J and stop the pump at any desired position. The syringe is held in place by the lucite block H. Two cylindrical lucite plugs attached to the holder project through holes in the pump mounting plate. The holder is removed by lifting vertically. It is convenient to make several holders with identical mounting plugs to accommodate several syringe sizes. About 10 seconds are required to remove the syringe, refill

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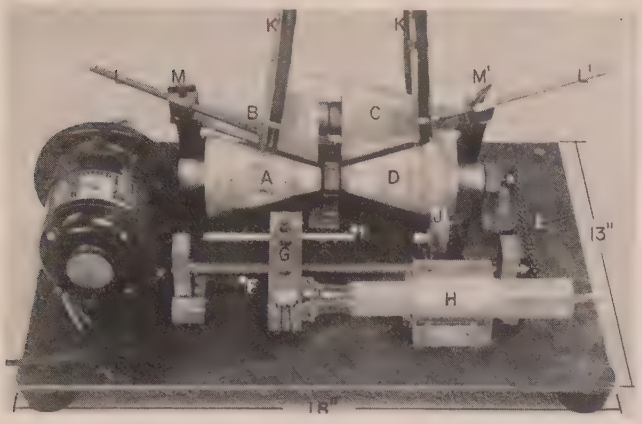

FIG. 1. Front view of infusion pump.

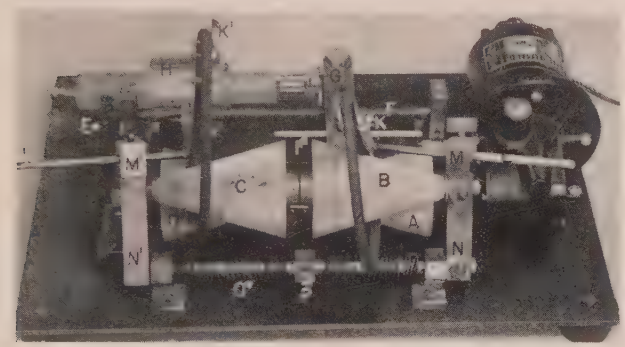

FIG. 2. Rear view of infusion pump.

it and begin the infusion again. The syringe drive can be easily modified to handle multiple syringes simultaneously.

Several factors influence the rate of delivery from the syringe: 1) speed of the gear drive from the motor; 2) relative sizes of the cones; 3) relative sizes of the sprockets in assembly E; 4) pitch of the threads on shaft F; and 5) volume delivered per unit of barrel length of the syringe. The present instrument has a motor drive speed of 11.5 rpm, 10-fold reduction or acceleration in the cone drives (100-fold total variation), 2-fold fixed reduction in the sprocket assembly, and 16 threads per inch on shaft F. Rate of delivery is therefore 0.50 to 50 ml per minute with a standard 50 ml syringe and 0.15 to 15 ml per minute with a 10 ml syringe. Rate of delivery for a given syringe can easily be calibrated by marks on rods L and L′. Constancy and reproducibility of infusion rate was checked by timing over 6 runs the delivery of 10 ml (10 ml syringe) at an arbitrary setting. With the setting unchanged, the range of times was 3.117 to 3.150 minutes. When rods L and L′ were moved and returned to the setting the range was 3.107 to 3.177 minutes. With the setting unchanged the range was 3.193 to 3.206 when fluid was delivered against a pressure of 100 cm of water.

The materials for this infusion pump cost approximately $60. No intricate machining is required, nor are the adjustments critical. Furthermore, variations in speed, range of variation, total capacity, etc. can easily be made by certain obvious alterations in the instrument which has been described.

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Synergistic Role of Prolactin in Response of Male Rat Sex Accessories to Androgen.* (23050)

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Huggins and Russell(1) were the first to point out that there is greater prostatic atrophy following hypophysectomy than following gonadectomy in the dog. Other workers(2,3) have reported that hypophysectomized male rats showed less prostatic response to androgen than did animals that had undergone gonadectomy alone. Moreover, several investigators(2,4-8) have concluded that administration of various pituitary factors enhances the response of the ventral prostate and other sex accessories to androgen (or to interstitial cell-stimulating hormone, ICSH, in the presence of the testis). The conclusions derived from these investigations have often been conflicting, and little control has been exercised over the many variables (purity and dosages of pituitary hormones, age and operative condition of rats). Furthermore, in regard to enhancement of ICSH effects, conclusions obtained in this instance do not indicate whether or not the observed effects on the accessories of the administered pituitary hormone are the result of a direct effect upon the sex accessories (*i.e.*, synergism with androgen) or of an increase in androgen secretion by the testis (*i.e.*, synergism with ICSH) or of both effects.

The object of the current study was to ascertain the effects of lactogenic hormone (prolactin, LTH) on response of sex accessories of hypophysectomized-castrate male rats to administration of testosterone propionate (TP). The dose of TP employed was one which caused doubling of the control ventral prostate weights, a response typical of that which follows ICSH administration(8). The dose of LTH was similar to that used by Segaloff(8) and shown by him to modify the response to ICSH administration. The effect of pituitary growth hormone (somatotropin, STH) was also investigated, but only at dose levels selected to control for a possible contamination by STH in the LTH preparations. The use of immature rats at a time long enough after castration and hypophysectomy to eliminate any effects contributed by residual circulating hormones permitted us to determine stimulation of growth, rather than maintenance of development already attained (7).

Materials and methods. 144 male Sprague-Dawley rats were castrated at 28-30 days of age and hypophysectomized 2 days later. Beginning 11 days after hypophysectomy, single daily hormone injections were given for 5 days. Aqueous solutions of LTH and of STH were administered intraperitoneally; TP in sesame oil was injected subcutaneously. Table I lists the various groups of animals and the amounts of hormones used. Rats of Series I and II were obtained from a different source than those of Series III and IV. The LTH was prepared from ovine pituitaries by previously published methods(9,10) and possessed an estimated potency of 35 I.U./mg; the STH was prepared from beef glands by the method of Li(11). On day following 5th injection, rats were sacrificed by neck fracture; ventral prostate, anterior prostate ("coagulating gland"), and seminal vesicles were removed, freed of the bulk of their secretions, and weighed on a 0-50 mg Roller-Smith balance. The *sella turcica* was inspected for evidence of pituitary fragments. The ventral prostate was bisected: one portion was reweighed and immediately frozen on dry ice for later determination of alkaline phosphatase activity(12); the other portion was fixed in Bouin's fluid, serially sectioned

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TABLE I. Effect of Testosterone Propionate (TP), Lactogenic Hormone (LTH) and Growth Hormone (STH) on Sex Accessories of Hypophysectomized, Gonadectomized Male Sprague-Dawley Rats.

Series	Daily dose, μg			No. of rats	Body wt, g		Wt, mg§			Glandular tissue in ventral prostate, %§
	TP	LTH	STH		Initial	Final	Ventral prostate	Anterior prostate	Seminal vesicle	
I	0	0	0	10	77	76	6.7 ± .4	2.1 ± .1	5.9 ± .4	
	0	150	0	7	82	88	7.8 ± .6	2.4 ± .1	7.4 ± .4†	
	0	0	7.5	10	70	69	5.9 ± .3	1.4 ± .1	4.9 ± .3	
II	25	0	0	14	83	84	12.4 ± .5	3.7 ± .3	13.9 ± .9	64.2 ± .7 (8)‖
	25	150	0	12	84	86	16.0 ± .7‡	4.6 ± .6	17.3 ± 1.4*	70.3 ± .8‡ (8)
III	25	0	0	8	71	75	15.4 ± .9	4.5 ± .2	15.7 ± .7	66.3 ± 1.1 (8)
	25	150	0	9	70	74	16.6 ± .7	5.2 ± .3	18.9 ± 1.0†	70.4 ± .8‡ (9)
	25	0	7.5	9	69	75	17.7 ± .7	5.2 ± .3	21.3 ± 1.1‡	67.1 ± .9 (9)
IV	25	0	0	13	79	79	15.5 ± .9	5.5 ± .4	22.3 ± 1.4	50.6 ± 1.0 (13)
	25	150	0	10	82	84	17.1 ± 1.1	7.0 ± .5*	27.1 ± 1.5	55.6 ± 1.1‡ (8)
	25	300	0	11	83	85	18.4 ± 1.1	8.3 ± .9†	29.7 ± 2.1‡	
	25	0	3.8	11	83	87	18.7 ± 1.1*	6.9 ± .4*	25.2 ± 1.3	
	25	0	7.5	10	80	84	17.8 ± 1.0	6.3 ± .6	26.1 ± 1.6	51.6 ± 1.3 (10)
	25	150	7.5	10	84	88	21.0 ± 1.1‡	8.1 ± .5‡	32.5 ± 1.2‡	55.7 ± 1.0‡ (9)

* $.02 < p < .05$. † $.01 < p < .02$. ‡ $p \leq .01$. All "p" values were calculated with respect to the first group in each series.

§ Mean ± S.E. of mean. ‖ No. of ventral prostates examined histometrically.

in paraffin, and stained with hematoxylin and eosin or Mallory-Heidenhain stain. In Series II and III, histometric analysis of sections of the ventral prostate was carried out by means of a technic modified from Eränkö(13). The sections from Series IV were studied histometrically with a synchronous scanning device designed by one of us (M.D.C.). In this apparatus, time is used as a measure of distance as the magnified image moves at constant speed past a crosshair superimposed upon the field. This scanning method allowed a more random and hence more objective selection of sections for measurement than the method employed in the earlier series.

Results. In preliminary studies with hypophysectomized - gonadectomized Long-Evans rats, the addition of 150 μg LTH to daily doses of 25-500 μg TP produced no significant increases in the weights of the sex accessories over those of rats receiving TP alone. However, Sprague-Dawley rats subjected to the same treatment showed significant responses (see Series II), and this strain was chosen for further investigation.

Table I summarizes the results. Neither STH nor LTH alone had any stimulatory effects on the ventral or anterior prostates, but LTH apparently elicited minimal growth on the part of the seminal vesicles. TP alone resulted in at least a doubling of the weight of all structures studied over those of controls injected with sesame oil. LTH administered with TP produced a significant increase in the weight of the ventral prostate in Series II, an increase which was accompanied by a concomitant significant increase in the proportion of glandular tissue in this organ.

Since the LTH preparation employed in Series I and II contained a small amount of STH (as indicated by the tibia assay method (14)), an additional group, injected with 7.5 μg STH daily, was introduced to control for a maximal (5%) possible contamination of the LTH with STH. Administration of LTH or STH in Series III resulted in a significant weight increase only in the seminal vesicles; however, LTH effected an increase in the amount of glandular tissue in the ventral prostate, an increase not seen with STH alone.

In the last series (IV), an LTH preparation was employed at 2 dose levels, which, when assayed for growth hormone activity at a total dose of 4 mg, gave no indication of such activity, indicating that any contamination was less than 0.25%. In addition, 2 dose levels of STH were used. Finally, a group was included receiving TP, LTH and STH together, in order to determine whether the in-

dicated individual synergistic effects were additive. The highly purified LTH preparation caused small increases in ventral prostate weight (also evident in Series III) that were not significantly different at either dose level from those observed in the rats treated with TP alone. Significant weight increases were observed in the anterior prostates of both LTH groups and in the seminal vesicles of the groups receiving the dose of 300 μg daily. STH produced no consistent organ weight increments. When 150 μg LTH and 7.5 μg STH were combined with 25 μg TP, highly significant large weight increases (more than 35% above the TP control group) were observed in the ventral prostate, anterior prostate, and seminal vesicles. Significantly more glandular tissue was seen in the ventral prostate in the presence of LTH than in its absence. STH did not have this latter effect.

LTH was shown to have no effect on alkaline phosphatase activity as determined in ventral prostate samples from TP-treated and LTH + TP-treated rats in Series II and III.

Discussion. The sex accessories of rats of the Long-Evans strain in our experiments and in those of Lostroh and Li(7) proved to be unresponsive to the administration of pituitary hormones, even though this strain appeared as sensitive as the Sprague-Dawley to injected androgen after gonadectomy and hypophysectomy. Hence, the results herein reported must be considered as referring to rats of the Sprague-Dawley strain.

Neither LTH nor STH alone was capable of producing significant increases in ventral prostate weight. In only one series (II) did combined LTH and TP administration result in ventral prostates significantly larger than those produced by TP injection alone, and in this series the mean prostate weight of the TP-injected control group was lower than in the other series, in which the animals were obtained from a different source. Nevertheless, despite the failure of LTH to produce a consistent significant increase in the weight of the ventral prostate, a significant increase in the proportion of glandular tissue in that organ was revealed by histometric analysis. Although the combined administration of STH and TP in Series III produced ventral prostate weights equal to or larger than those seen following treatment with LTH, no concomitant increment in glandular tissue was observed with the combined treatment in this series or in Series IV. Neither pituitary hormone, either alone or in conjunction with TP, produced a significant increase in anterior prostate weight in the earlier series. However, with the highly purified LTH of Series IV, significant augmentation of weight of this organ was observed at both dose levels employed. The response to STH, on the other hand, was not so marked and even less regular in occurrence. The seminal vesicle appeared to be the sex accessory in which increase in weight resulted most consistently from the combined administration of LTH and TP. An effect of LTH on the seminal vesicles of gonadectomized rats has been reported previously by Pasqualini(5). In one experiment when STH was administered with TP, a synergistic response was obtained.

The greatest responses to androgen were manifested by all 3 accessories after simultaneous treatment with STH and LTH. In all cases the difference in organ weights between the triply injected animals and those receiving TP alone was found to be significant at "p" values of less than .001. A significant increase in glandular tissue in the ventral prostate was also seen ($p<.01$).

The results indicate that either LTH or STH, at the dose levels employed, is capable of exercising only a minimal degree of synergism with androgen in stimulating male sex accessory growth. This trend of minimal synergism is evident throughout almost all the data; however, statistical evaluation of the data from any single series only occasionally reveals differences that are significant. However, marked synergism, of definite statistical significance, was obtained after simultaneous treatment with all 3 hormones; thus, it appears that both LTH and STH as entities are needed for the optimum synergistic response. Li(15) has recently described STH as a general metabolic hormone, and indeed the increased responsiveness of sex accessories in the presence of STH, and pos-

sibly of LTH, could be viewed in terms of improvement in the general physiologic state of the organism. It would appear, however, that LTH and STH act by different mechanisms, since the former, but not the latter, consistently produces an increase in the proportion of glandular tissue in the ventral prostate.

Summary and conclusions. Lactogenic hormone (LTH) alone or together with testosterone propionate (TP) produced a significant increment in the weight of the seminal vesicle. When given with TP, LTH produced an increase in the amount of glandular tissue in the ventral prostate. Growth hormone (STH) alone or together with TP did not produce any consistently significant increase in the weight of the accessories. When TP, LTH and STH were administered simultaneously to the same test animals, the weight response of all accessories was significantly greater than that to androgen alone. The data are interpreted as indicating that a synergism among the 3 hormones may operate to stimulate sex accessory growth in the male Sprague-Dawley rat.

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Effects of Cortisone and Hydrocortisone on Sodium Excretion in Adrenalectomized Rats. (23051)

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Sodium retention with cortisone and hydrocortisone is readily demonstrated in man (1-4). In adrenalectomized rats, commonly used for steroid studies, retention of sodium has not been clearly seen. Singer and Venning(5) in fact observed sodium loss with cortisone, but were unable to obtain a graded response. Results of Johnson(6) and of Dorfman(7) showed increased excretion of the ion with small doses of hydrocortisone, and no effect with larger doses. In our experience, cortisone has caused a loss of sodium with low doses and retention with high doses. It should be carefully noted that a fixed time-interval of urine collection was used for all the foregoing laboratory studies.

Sodium response in rats was therefore further investigated as a function of time and dose following cortisone and hydrocortisone administration. Information of this kind is useful for careful evaluation of sodium-retaining activity in structurally-related steroids. The present report summarizes our data.

Material and methods. Exp. I. Large

TABLE I. Effects of Large Doses of Cortisone and Hydrocortisone on Sodium Excretion in Consecutive Samples of Urine.

Treatment†	mg/rat	No. of animals	μEq sodium/105 min. period (mean ± S.E.) 1st	2nd	3rd	4th
CO		26	96 ± 10	63 ± 5	41 ± 4	30 ± 3
E	.5	12	86 ± 14	81 ± 16	92 ± 13*	98 ± 15
	1.0	12	91 ± 11	61 ± 6	105 ± 11*	125 ± 12*
F	.5	10	79 ± 7	46 ± 8	64 ± 7*	92 ± 10*
	1.0	10	80 ± 8	37 ± 5*	56 ± 7	86 ± 12*

* P = .05 or less. † CO = Corn oil; E = Cortisone; F = Hydrocortisone.

doses of cortisone and hydrocortisone were given to adrenalectomized rats, and the effects on sodium excretion determined in consecutive samples of urine. This study provided data on time-response relationships with the steroids. Sprague-Dawley male rats (140 to 175 g) adapted to laboratory conditions for about a week were starved overnight. Animals were tube-fed with 10 ml of a suspension containing 3.8 g rat food (Rockland Farm) at 9 a. m. and 4 p. m. for 2 consecutive days. Bilateral adrenalectomy was performed under ether anesthesia at 11 a. m. on the 2nd day of feeding. Tap water was provided *ad libitum* during the tube-feeding regime. Doses of 0.5 and 1 mg of steroids in 0.2 ml corn oil (Mazola) were subcutaneously injected on the morning of the 3rd day. Control rats received the same volume of oil. Each animal was forced to void by application of pressure to the bladder, and then placed in an individual metabolism cage. Following injection, 4 consecutive samples of urine were collected at intervals of 105 minutes. Complete specimens of urine were obtained at the end of each period by manipulation of the bladder and by rinsing cages with distilled water. Sodium concentration was determined with the Beckman flame photometer for calculation of total excretion per period. Relative differences in excretion between control and treated animals were computed for each period to examine the influence of the steroids. *Exp. II.* This study was to determine the influence of dose (0.0625 to 0.5 mg) of hydrocortisone upon time-response relationships. Detailed procedures were similar to those described for Exp. I.

Results. The effects of 0.5 and 1 mg cortisone and hydrocortisone on sodium excretion are summarized in Table I. Changes in sodium excretion compared with the control group are plotted in Fig. 1. In general, sodium retention preceded a diuretic phase with both 0.5 and 1 mg doses. Initial retention was small with cortisone (E), but by virtue of a strong diuresis during periods 3 and 4, sodium loss resulted for the total period of study. Both groups receiving hydrocortisone (F) responded initially with relatively greater retention of sodium and subsequently with increasing diuresis. The net effect with F is likewise sodium loss, but is less pronounced than that of E.

For both steroids, 1 mg seemed more effective than 0.5 mg in delaying onset of sodium diuresis. Inspection of curves indicates that there is more prolonged duration of sodium retention with increasing doses. This effect is exemplified by Fig. 1 which shows that length of retention phase varies directly as dose of E. A similar relationship between dose and duration of retention phase

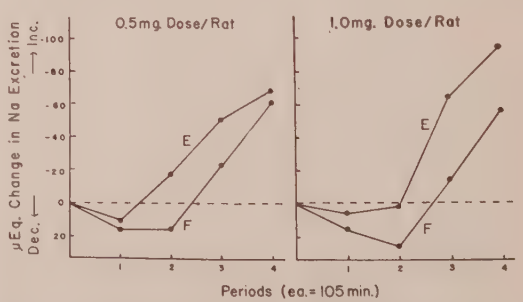

FIG. 1. Relative changes in excretion pattern of sodium following inj. of large doses of cortisone (E) and hydrocortisone (F). Values above control line (– – – –) represent increased excretion and values below, retention.

TABLE II. Effects of Graded Doses of Hydrocortisone on Sodium Excretion in Consecutive Samples of Urine.

Treatment†	mg/rat	No. of animals	μEq sodium/105 min. period (mean ± S.E.) 1st	2nd	3rd	4th
CO		10	65 ± 8	50 ± 7	51 ± 9	43 ± 11
F	.0625	10	81 ± 7	66 ± 7	66 ± 9	69 ± 8
	.125	10	59 ± 7	65 ± 10	76 ± 8	89 ± 6*
	.25	10	58 ± 6	51 ± 7	82 ± 6*	100 ± 9*
	.5	9	56 ± 7	42 ± 7	80 ± 12	102 ± 12*

* P = .5 or less. † CO = Corn oil; F = Hydrocortisone.

is seen with curves of F, but the differences are not so marked.

Data concerning the temporal phase of sodium retention as a function of dose of F were obtained in Exp. II (Table II). The total time required for the excretion rate of sodium to equal that of the controls was estimated from the time-response curves. A value of zero was assigned to the group receiving 0.0625 mg F, since no retention of sodium occurred in any of the 4 periods of study. Curve I of Fig. 2 illustrates relationship between dose of steroid (log scale) and duration of sodium retention. Results with 0.5 and 1 mg F from the previous experiment are shown as Curve II in the Figure. This method of evaluation is only roughly quantitative, but it demonstrates the direct relation between duration of retention phase and dosage. Examination of data in the Table reveals, on the other hand, that degree of sodium retention appears relatively less dependent upon dose of these steroids.

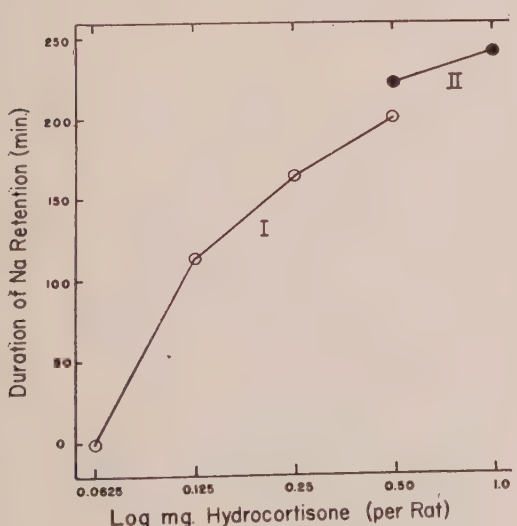

FIG. 2. Relationship between duration of sodium retention and dose of hydrocortisone (log scale). Curves I and II represent separate studies.

Discussion. Our results show that large doses of E and F cause a biphasic effect on sodium excretion. In general, a small retention precedes a strong diuresis, the net effect being sodium loss. Smaller doses hasten onset of secondary excretion phase and decrease duration of sodium retention. It is evident that entirely different results are possible by varying the time-interval of urine collection. Increased excretion will be the predominant phenomenon over a long period, because the sample includes a large segment of secondary diuretic phase. With a shorter collection period, no change will occur because retention is opposed by the same degree of sodium diuresis. Finally, only sodium retention will be found with relatively brief collection. A careful evaluation requires the testing of a wire range of doses to discover secondary effects which may be operating.

Our results indicate that E and F are capable of retaining sodium in adrenalectomized rats, as they do in man(1-4). However, degree of retention appears weak and only transient in comparison with the secondary excretion phase, and consequently is demonstrable only with large doses under special conditions of urine collection.

The biphasic nature of sodium excretion does not seem to be confined to the actions of E and F. An early report by Harrop(8) shows that α-estradiol caused an initial retention, then a loss of sodium in normal dogs maintained with constant fluid and mineral intake. Simpson and Tait(9) have discussed

a biphasic response with DCA, which elicited a net loss of sodium in adrenalectomized rats. The data of Forsyth(10) suggest the strong influence of this phenomenon with DCA, by showing increased excretion with small doses, and retention with larger doses in adrenalectomized mice.

Summary. 1. The influence of time and dosage upon sodium excretion by rats was investigated after cortisone and hydrocortisone administration. 2. Results of time-response studies show that large doses cause initially weak and transient retention of sodium and subsequently strong excretion of the ion. Smaller doses reduce progressively duration of retention phase and hasten onset of secondary diuresis. 3. Two factors appear to influence the response of sodium excretion. During a fixed time-interval of urine collection, increasing doses cause excretion, no change and retention of sodium. Similar changes in excretion of the ion are seen with a given dose when collection interval is progressively shortened.

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Comparison of Tissue and Plasma Thromboplastic Activities.* (23052)

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Since introduction of the thromboplastin generation test by Biggs and Douglas(1) a great many studies have been carried out dealing with defective formation of "plasma thromboplastin" in various coagulation abnormalities. This plasma thromboplastin is defined as activity developed in a dilute mixture of adsorbed plasma ($Al(OH)_3$ or $BaSO_4$), serum, platelets and calcium. When samples of such a mixture, together with additional calcium, are added to normal plasma "thromboplastic" activity develops during 5-minute incubation as clotting times decrease from over a minute to approximately 10 seconds. Relatively little attention has been paid to the activity of fully formed normal plasma thromboplastin in the clotting mechanism. A distinct difference between plasma and tissue thromboplastin was demonstrated by Gollub, Ulin and Black who showed that the bacterial enzyme thromboplastinase would inactivate tissue thromboplastins, but not plasma thromboplastin, although it did have some inhibitory effects in early stages of plasma thromboplastin generation.

The simple experiments presented in this study show certain striking differences between tissue and plasma thromboplastin.

Materials and methods. Tissue thromboplastin: Human Brain: 300 mg acetone-dried human brain powder suspended in 5

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ml 0.85% sodium chloride, and incubated 10 min. at 37°C. The material was allowed to sediment for 30 min. and the supernatant employed. Rabbit Tissue: Simplastin (kindly supplied by Warner-Chilcott), used as directed, without additional calcium. *Plasma thromboplastin*: 1 ml normal platelet suspension plus 1 ml normal $BaSO_4$ plasma (dil. 1-5) plus 1 ml normal oxalated serum (dil. 1-10) plus 1 ml 0.025 M $CaCl_2$. All tests were carried out in 15 min. after the mixture had been allowed to activate for 8 min. The same clotting time (10 sec.) on normal citrated plasma was obtained at beginning and end of experiment. *Test plasmas*: Unless otherwise stated all normal and patients' plasmas were citrated and had been stored frozen 2 weeks to 6 months. The PTA deficient plasma was obtained from patient previously described (A. A.(2)) who was seen through the courtesy of Dr. Robert L. Rosenthal. The Hageman factor deficient plasma was obtained from a patient, also previously described (M.R.(3)), and studied through the courtesy of Dr. Oscar Ratnoff. The proaccelerin deficient plasma was from a girl previously described (D.H.(4)). The proconvertin deficient plasma, lacking "Stuart factor," was a lyophilized preparation supplied through the kindness of Dr. John Graham. This patient has been described by one of us(5) and by Dr. Graham(6). The plasma designated Dicumarol was from a 63-year-old man on long-term, poorly controlled therapy who showed hemorrhagic symptoms. The patient with vit. K deficiency was a 4-month-old infant with hemorrhagic symptoms one month and signs of intracranial hemorrhage 2 days. A prompt response followed vit. K therapy. The patient used as example of liver disease suffered from severe post-hepatitic cirrhosis confirmed at autopsy. The heparinized plasma was obtained from normal subject one hour after 50 mg heparin was administered i.v. Recalcification times were carried out in duplicate by adding 0.1 ml thromboplastin or saline, simultaneously with 0.1 ml 0.02 M $CaCl_2$ to 0.1 ml plasma at 37°C. Results of other test procedures are from the initial patient workup by our standard methods(7,8).

TABLE I. Recalcification Times.

Type of plasma	Recalcification times (sec.) in presence of			
	Saline	Rabbit tissue	Human brain	Plasma thromboplastin
Normal	145	12.2	15.0	10.0
" platelet-poor	390	12.0	14.2	10.0
AHF Deficient	1,350	12.2	14.2	9.8
PTC "	1,620	12.0	17.5	10.1
PTA "	570	12.0	15.1	10.4
Hageman " factor	960	13.0	16.2	9.8
Proaccelerin "	820	32.2	70.0	9.3
Proconvertin " (Stuart)	745	53.6	117.0	9.2
Mixed deficiencies				
Dicumarol	695	44.5	98.5	39.5
Vit. K deficient	3,600	33.0	69.0	32.0
Liver disease	695	30.8	60.2	16.8
Normal— heparinized	>1,000	14.8	22.2	40.0

Results. The Table shows recalcification times of various plasmas in the presence of saline, rabbit tissue, human brain and plasma thromboplastins. All saline times are significantly longer than that of normal plasma indicating that platelets, AHF (anti-hemophilic factor), PTC (plasma thromboplastin component), PTA (plasma thromboplastin antecedent), Hageman Factor, proaccelerin and proconvertin (Stuart) are all factors essential to normal coagulation. This was borne out by initial examinations on these patients, all of whom showed abnormal thromboplastin generation curves as well as other coagulation abnormalities. The patients suffering from mixed deficiencies: dicumarol intoxication (prothrombin 2%, proconvertin 1%, PTC 20%), vit. K deficiency (prothrombin 5%, proconvertin 5%, PTC 15%) and hepatic cirrhosis (prothrombin 26%, proconvertin 3%, proaccelerin 39%, and PTC 10%), also showed abnormal thromboplastin generation curves on the initial examinations and prolonged recalcification times.

Human brain and rabbit tissue thromboplastin times were normal in platelet, AHF, PTC, PTA and Hageman factor deficiencies, but markedly prolonged in proaccelerin, proconvertin and the mixed deficiencies, and in

heparinized plasma. The plasma thromboplastin times were normal in all except the mixed deficiencies in which prothrombin itself was low, and in heparinized plasma.

In another experiment plasma thromboplastin was prepared as usual and then centrifuged at 20,000 g for 30 min. When the sediment was resuspended in fresh saline and calcium, it had almost all the activity of the original mixture, when tested against either normal or deficient plasmas. If the sedimented material was suspended in saline, without calcium, no clot was formed when it was added to plasma but a clot did form when additional calcium was simultaneously added.

Discussion. From the foregoing experiments it appears that "plasma thromboplastin" is a complex, sedimentable at high speed centrifugation, separable from calcium ion, inhibitable by heparin, which can substitute for the activities of platelets, AHF, PTC, PTA, Hageman factor, proaccelerin and proconvertin (Stuart), and which, in the presence of calcium, appears to convert prothrombin to thrombin directly. This latter premise is based upon the observation that only the plasmas low in prothrombin gave prolonged plasma thromboplastin times. Recent experiments suggest the possibility that the SPCA deficient patient of Alexander(9) and the hypo-proconvertinemic patient described herein and previously(5), deficient in the so-called "Stuart Factor" (Hougie and Graham(6)), may suffer from different deficiencies although both show prolonged prothrombin times and abnormal proconvertin levels as assayed by the Owren method. Alexander's patient is said to have a normal thromboplastin generation, and therefore it is possible that this factor is not necessary for plasma thromboplastin generation, but acts at a later stage. If this is the case it would seem likely that this patient's plasma might have a prolonged plasma thromboplastin time.

Brain thromboplastin and plasma thromboplastin are similar in their abilities to shorten recalcification times in platelet, AHF, PTC, PTA and Hageman factor deficiencies. Both give long recalcification times in the presence of prothrombin deficiencies. Both appear to be inhibited by heparin, although these experiments do not clearly differentiate this effect from an anti-thrombic one. Brain and plasma thromboplastin differ strikingly when tested against proaccelerin and proconvertin deficient plasmas, for only plasma thromboplastin is able to clot these plasmas in normal times. That this effect is not simply due to carrying over of these factors in plasma-thromboplastin mixture is shown by the observation that after high speed centrifugation and resuspension of the "plasma thromboplastin" it is still active in these deficiencies.

Summary. 1. A lengthening of saline-recalcification time beyond normal occurred in plasma deficient in any one of the following: platelets, AHF, PTC, PTA, Hageman factor, proaccelerin, or proconvertin. It also occurred in certain acquired multiple factor deficiencies, and in heparinized plasma. 2. Brain thromboplastin recalcification times were normal in platelet, AHF, PTC, PTA and Hageman factor deficiencies, but prolonged in proconvertin or proaccelerin deficiency or heparinized plasma. 3. Plasma thromboplastin recalcification times were normal in these same deficiencies and, in addition, in proconvertin or proaccelerin deficiency. This latter activity seemed due to incorporation of (pro) convertin and (pro) accelerin into the plasma thromboplastin complex, rather than to any free proconvertin and proaccelerin in solution. 4. Formed plasma thromboplastin is inactive in absence of optimal calcium concentration.

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Cardiac Retroperfusion with Induced Asystole for Open Surgery upon the Aortic Valve or Coronary Arteries.* (23053)

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The concomitant use of a pump-oxygenator (1,2) for circulatory support during total by-pass of the heart together with retrograde perfusion via coronary sinus permits open surgery on the ascending aorta and aortic valves for periods of 15 to 30 minutes. This technic has been tested experimentally and successfully applied subsequently in man(3,4). Retrograde perfusion via coronary sinus of arterial blood supports myocardial activity and prevents coronary air embolism during interval that the ascending aorta remains open. As a rule almost no coronary venous drainage occurs from the right ventricle into the coronary sinus in the dog. Therefore, virtually no retroperfusion of this myocardial area takes place. Despite this fact the canine right ventricle appears to tolerate 15 to 20 minutes of by-pass assisted by retroperfusion, because cardiac workload is almost completely eliminated, and the left ventricular muscle remains oxygenated. In man the anatomical pattern of coronary veins usually permits more adequate retroperfusion of the right heart through the coronary sinus. Previously we reported on retroperfusion technic (4). There were 12 long-term survivals among 16 dogs, submitted to this technic, for intervals of 15 to 20 minutes. Only one of 4 deaths was related to cardiac complication. Four additional animals were submitted to 30 minutes of retroperfusion. Three of them died apparently from irreversible fibrillation appearing at termination of the retroperfusion and presumably induced by intolerable state of right ventricular hypoxia. Blanco *et al.* (5) reported survivals in dogs after retroperfusion of coronary veins up to 7¼ minutes.

This duration of retroperfusion, safe for dogs and probably for man, must certainly be extended to 30 minutes or more if we are to realize its full usefulness. Therefore, we have tested the potential advantages of combining retroperfusion of the coronary sinus with asystole induced with potassium citrate, hoping thereby to reduce further myocardial oxygen consumption. Also, the advantages of working in a completely still operative field would be realized. And finally, both experimentally and clinically it has been found dangerous to attempt to re-start a deliberately arrested heart by forward coronary perfusion unless vents into the left ventricle and left atrium have been provided to keep those chambers decompressed. In patients without septal defects it may not be technically feasible to establish these vents; and therefore, a method for restoring the beat (by means of retroperfusion) with the aorta still open becomes valuable. The results of these studies comprise the subject matter of this paper.

Method of study. Forty dogs had their heart and lungs by-passed utilizing a pump oxygenator(1,2). They were subdivided into 4 groups. *Group I* consists of 10 dogs sub-

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† National Heart Trainee, U.S.P.H.S.

mitted to retrograde perfusion of coronary sinus for 30 minutes. *Group II* (10 dogs) had retroperfusion for 30 minutes in combination with asystole induced by potassium citrate. The 10 dogs in *Group III* experienced potassium citrate achieved asystole only, for 15 to 30 minutes. The final 10 dogs in *Group IV*, acute experiments, were studied for metabolic changes and coronary flow distributions during retroperfusion with and without potassium citrate provoked asystole. Two methods for *perfusion of coronary sinus* were tested. In one, arterial blood was tapped off the main arterial limb and carried through a Y connector. In the other, arterial blood from oxygenator reservoir was propelled by separate pump directly into the cardiac venous system. A bifurcating arterial limb (first method) was used for survival experiments. To prevent perfusion of coronary veins at too high pressures, an adjustable clamp was placed on catheter leading into coronary sinus. The tension distal (cardiac) to this clamp was then monitored through a strain gauge and appropriate recording apparatus. We sought to keep it at mean pressure of 30 to 40 mm Hg. Earlier experiments(4) suggested that these pressures were well tolerated by the coronary veins and that they provided for adequate retrograde flow. The coronary sinus was perfused directly from a separate pump in acute metabolic studies so that a more exact coronary flow could be established in each experiment. To cannulate the coronary sinus a #14 polyethylene catheter with a firm plastic adaptor, ridged on end, was placed into the coronary sinus and anchored there either by silk ligature placed around the sinus from outside the heart, or by a purse-string suture about the coronary sinus ostium working from within the right atrium. The latter seems to be the more suitable method and has been utilized for clinical cases. *Cardiac Asystole*: When retroperfusion was combined with potassium citrate asystole, this was induced after the technic of Melrose(6). A 2.5% solution of potassium citrate diluted in the animals' arterial blood was injected rapidly into the ascending aorta until the heart ceased to beat and the aorta cross clamped just above the coronary artery orifices to prevent any perfusion via this route.

TABLE I. Total Cardiopulmonary By-Pass Procedures with Retroperfusion of Coronary Sinus and K Citrate Asystole.

Procedure	Vent. fib., %	Card. mort., %	Comments
A Retroperfusion, 30 min.	40	40	All 4 fatalities had irreversible fibrillation at end of retrop'rf'sion
B *Idem* with K citrate asystole, 10-30 min.	40	20	2 fatalities 2 hr postoperative
C K citrate asystole alone, 15-30 min.	80	90	1 dog had irreversible fibrillat'n; remaining deaths within 12 hr postoperative

In recent experiments (not herein reported) it has been found technically feasible to arrest the heart retrogradely by slowly injecting 25% potassium citrate solution into the arterial blood in the retroperfusion catheter. When arrest occurs, this injection is stopped and the retroperfusion catheter is cross-clamped. The heart is restarted by re-initiating the retroperfusion.

Results. The results for 10 dogs retroperfused for 30 minutes are presented in Table I. Four animals fibrillated at end of retroperfusion, and it was not possible to defibrillate any.

The data in 10 experiments wherein asystole was achieved and in which there was standstill varying from 6 min. to 30 min. with concomitant retrograde perfusion of the coronary sinus of 30 min. duration in all are presented in Table I(B). We noted that when the hearts did resume their beat at the end of the experiments, that this was solely a left ventricular contraction. The poorly perfused right ventricles usually remained quiescent at first, and then gradually and progressively acquired a satisfactory beat. Two of these 10 dogs died from cardiac causes during the immediate experiment. In these animals the period of induced asystole was 15 min. and 25 min., respectively. Four additional dogs succumbed several days postoperative from non-cardiac complications.

TABLE II. Comparison of Distribution of Retroperfusate. Dog #1855 had retroperfusion alone and dog #1494 had retroperfusion in combination with K citrate asystole.

Mean pressure in cor. sinus, mm Hg	Dog No. and procedure	Flow in cc/min.		
		Cor. sinus input	L. cor. artery outflow	Right thebesian outflow
20	1855, retro. alone	16	5	8
	1494, retro. with K citrate	30	14	15
30	1855	32	9	20
	1494	33	16	16
40	1855	42	12	28
	1494	35	16	18
50	1855	64	18	42
	1494	44	17	24

These deaths are probably ascribable to use of clean but not sterile technic. The late incidence of severe infections was substantial. Of 10 dogs (with cardiopulmonary by-pass) submitted to potassium citrate induced asystole for 15 to 30 minutes (Group III, Table I(C)) only one survived for a long term. All of the deaths in this group occurred within 14 hours postoperative.

Table II lists distribution of retrograde flow at different coronary sinus pressures. A representative study of retroperfusion alone has been compared with a comparable study utilizing both retroperfusion and cardiac asystole. The retrograde flow from the left coronary ostia was approximately the same in both experiments. A high volume flow from the coronary sinus into right ventricle via thebesian veins was revealed in both animals.

Certain aspects of cardiac metabolism were studied in 5 dogs subjected to retroperfusion alone, and in a similar number of dogs submitted to combination of retroperfusion and potassium citrate induced asystole. Mean values of these data are presented in Table III. In all cases coronary sinus pressure was monitored at 40 mm Hg. Representative blood samples were drawn after 15 minutes of retroperfusion. Apparently retroperfused hearts utilized oxygen, glucose, and lactate in a manner similar to that of normally perfused hearts but in substantially lower amounts. The oxygen consumption of retroperfused left ventricle was approximately one-third that of normally perfused left ventricle in a by-passed state. This is probably a reflection of the diminished amount of retrograde flow, which is approximately one-third the volume of normal forward coronary blood flow. It was of interest to note that only a slight reduction in consumption of oxygen and glucose occurred in the retroperfused hearts, asystolic from an injection of potassium citrate.

Discussion. The canine heart is not anatomically well adapted to retrograde perfusion through the coronary sinus because the right ventricle receives little flow under these circumstances. Nevertheless, these canine hearts are capable of tolerating this predicament for periods up to 20 minutes. However, when the canine heart is subjected to 30 minutes of retrograde flow it is likely to fibrillate on termination of the run with a great likelihood of death then ensuing.

Perhaps addition of potassium induced citrate asystole to prolonged period of retroperfusion may offer some improvement in this death rate by placing the cyanotic right heart at greater rest. This is supported by the fact that the cardiac mortality after 30 minutes of retroperfusion alone was 40%; whereas, after 30 minutes of retroperfusion plus potassium citrate arrest, it was 20%.

Recently in a few experiments we have injected the sinoauricular node with xylocaine, instituted retroperfusion of the coronary sinus for one hour, and have maintained an excel-

TABLE III. Average Metabolic Values for Dogs Submitted to Retroperfusion Alone and Retroperfusion in Combination with Potassium Citrate Asystole.

Calculated per 100 g left ventricular wt/min.	By-passed heart with normal coronary flow	By-passed heart with retroperfusion of coronary sinus	
			and potassium asystole
	10 animals*	5 animals	5 animals
Flow	54.9 cc	19.0 cc	14.2 cc
Oxygen C†	7.18 "	2.62 "	2.03 "
Glucose C	3.7 mg	3.2 mg	2.4 mg
Lactate C	2.5 "	.66 "	.01 "

* Metabolic values in Column I were obtained as initial studies in the 2 groups.

† C = Consumption.

lent beat without fibrillation or other signs of cardiac arrhythmia.

To date the technic of total cardiopulmonary by-pass with retroperfusion of the coronary sinus has been applied to 8 patients(4). The first case was done over a year ago(3) and the patient is well at this time. The clinical lesions for which we have used retroperfusion were instances of aortic stenosis, and regurgitation, aortic-pulmonary window, ruptured sinus of Valsalva, and complete transposition of the great vessels. The longest clinical retroperfusion was 32 minutes, and in every patient a strong beat was maintained throughout the procedure with no instance of fibrillation during or after the retroperfusion. These clinical observations substantiate the anatomical fact that the human heart is more completely perfused than the dog's by a retrograde flow through the coronary sinus. On the basis of these experimental studies and the clinical observations mentioned above, the combination of retroperfusion and potassium asystole appears valuable for precise, direct vision reparative procedures upon the aortic valve or coronary arteries in a quiescent operative field.

Conclusions. 1. Only one of 10 dogs submitted to 15 to 30 minutes of potassium citrate produced asystole was a long-term survival (90% mortality). 2. Ten dogs were submitted to 30 minutes of retroperfusion of the coronary sinus, and 4 died from cardiac causes, a 40% mortality. 3. Ten additional dogs were subjected to combination of 30 minutes retroperfusion and potassium citrate provoked asystole. Two of the 10 died from cardiac complications, mortality 20%. 4. Certain aspects of cardiac metabolism in the retroperfused heart with and without potassium citrate induced asystole have been studied in 10 dogs. There appears to be an additionally reduced degree of metabolism under these latter circumstances.

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Failure to Transmit Human Nonbacterial Gastroenteritis to Cats.* (23054)

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Existence of acute infectious nonbacterial gastroenteritis of presumed virus etiology has been documented by epidemiologic observations(1-3) and by transmission of the disease to human volunteers(1,4,5) although no etiologic agent has been established in tissue culture or laboratory animals. Studies in collaboration with Gordon and Dorrance(4) demonstrated, by feeding stool supernates to volunteers, that there are at least 2 different types of such gastroenteritis. Because Yamamoto *et al.*(6) had reported transmission of human diarrheal disease to cats by feeding 2 to 5 ml of filtered supernatant of aqueous stool, aliquots of the same inocula used in human experiments with Gordon and Dorrance(4) were given to cats. The present re-

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port was prompted by recent editorial reference(7) to these cat experiments of Japanese workers, and records failure of inocula infectious for man to induce disease in cats.

Materials and methods. Two inocula were used. *Marcy inoculum* was obtained from an induced case (T.H.) of afebrile gastroenteritis on 7/3/50 and processed(1) by Gordon on 8/27/51. The watery stools from which the inoculum was prepared were collected after 6th human passage of the Marcy agent. Seven ml of inoculum induced disease in 14 of 16 volunteers in experiments done between December 1951 and June 1952(4). *FS inoculum* was processed from the stool of volunteer (Case E.E.) with induced febrile gastroenteritis, representing the first human passage(4). Two to 10 ml of the inoculum caused disease in 5 of 15 volunteers(4). Both inocula were stored at –70°C during 11 months between human and cat experiments. *Tryptose phosphate broth* was used as control inoculum. *Animals.* Cats were collected from semi-rural areas and housed in separate cages removed from general animal quarters. They were held for 8 or more days prior to inoculation. These measures were taken to avoid infectious feline enteritis or panleucopenia(8,9). On receipt, the cats' nails were clipped, each was weighed and bled 10 ml by intracardiac puncture, and total leucocyte count was done. Subsequently, for 6 to 12 days prior to inoculation, daily rectal temperatures, number and character of stools passed and gross food intake were recorded. Stools were counted and characterized as formed, soft, or loose by changing the drop pans each morning and afternoon. Under intraperitoneal nembutal anesthesia, 5 ml of appropriate inoculum was introduced by stomach tube. Observations of weight, appetite, stools, leucocyte count (weekly), and rectal temperature (now recorded twice daily) were continued.

Results. Exp. 1. On 12/2/52, 5 cats (1 male and 4 females; Group A, nos. 1, 3, 5, 7, 9) were given tryptose broth, and 5 cats (2 males and 3 females, Group B, nos. 2, 4, 6, 8, 10) were given FS inoculum. Observations made before and after administration of these

TABLE I. Intragastric Administration to Cats of Stool Supernates from Humans with Nonbacterial Gastroenteritis.

Animal		Before inoculation				After first† inoculum				After second† inoculum			
		Stools*			WBC	Stools*			WBC	Stools*			WBC
Group	No.	F	S	L	Wt, kg	× 1000	F	S	I.	Wt, kg	× 1000	F	S	L	Wt, kg	× 1000
A	1	11/12	2/12	19/12	2.6	14.2	4/6	1/6	9/6	2.6	8.6	5/6	5/6	0/6	2.8	nd
	3	14/12	10/12	8/12	2.7	14.4	2/6	5/6	4/6	3.1	10.9	3/6	4/6	5/6	3.0	12.0
	5	1/11	11/11	8/11	3.3	17.2	3/6	2/6	6/6	3.6	15.6	2/6	5/6	1/6	3.5	12.5
	7	9/12	6/12	10/12	2.6	23.3	2/6	6/6	4/6	2.8	18.0	4/6	5/6	0/6	2.8	22.7
	9	4/ 6	0/ 6	0/ 6	2.1	17.6	5/6	1/6	0/6	2.2	15.5	5/6	1/6	0/6	1.9	14.8
B	2	9/12	8/12	14/12	2.2	17.0	3/6	5/6	9/6	2.2	10.2	4/6	2/6	4/6	2.4	8.6
	4	11/12	5/12	11/12	2.4	14.8	3/6	3/6	8/6	2.5	17.3	0/6	11/6	6/6	2.6	17.7
	6	9/11	3/11	9/11	3.6	19.6	2/6	2/6	8/6	3.9	22.7	1/6	6/6	3/6	3.8	21.3
	8	5/ 6	0/ 6	0/ 6	2.6	22.0	4/6	2/6	2/6	2.8	13.2	5/6	5/6	0/6	2.5	nd
	10	2/ 6	3/ 6	4/ 6	2.5	19.1	1/6	4/6	4/6	2.0	17.7					

* Numerator = No. of stools. Denominator = No. of days observed. F = Formed; S = Soft; L = Loose.
† Inocula: A: First = broth; second = Marcy. B: First = FS; second = broth.

materials are summarized in Table I. No febrile responses were apparent in either group. During control period, it was noted that animal No. 10 ate poorly and exhibited weight loss of 0.4 kg in 7 days. FS inoculum provoked no change in number and character of stools, but because of continued anorexia and weight loss, this cat was not given a second inoculum.

Exp. 2. Since no apparent disease was produced, on 12/16/52, 2 weeks later, the cats were again bled and intubated. Group A was given Marcy inoculum; group B was given broth. Animal no. 7 showed an increase in temperature from baseline of about 102°F to 105°F 3 days after inoculation. None of the other 4 cats given Marcy supernate showed a febrile response. None developed diarrhea (Table I). With the exception previously noted, all animals maintained or gained weight. Leucopenia did not occur. Thus, there were no signs suggestive of infectious gastroenteritis of cats(8,9).

Discussion. Since simultaneous human volunteer experiments were not done, the question may be raised as to whether the material used was still infective for man at the time of inoculation into cats. Aside from difficulty of execution, such simultaneous human experiments were considered to be unnecessary because available data indicated that the agents survived storage at –70°C. Prior to the experiments mentioned(4), the fecal source of Marcy agent had been stored for 18 months; the processed inoculum had been stored for 6 months. Further, with aliquots of this same inoculum Gordon, Patterson, and Whitney(10) later induced disease in human volunteers on 4 occasions 3 to 5 months after the cat experiments. These investigators(10) achieved a 66% attach rate in man with a dose of 3.5 ml, 1.5 ml less than the amount given to the cats. As regards the FS agent, the original inoculum had induced disease in man after storage for 4 months. The material given the cats (first passage inoculum) had induced disease in man after 8 months storage. These data justify the assumption that the FS agent was still viable at the time of the cat experiments.

Yamamoto *et al.*(6) provoked diarrhea in all 6 cats given human stool supernate. The cats became ill 9 to 13 days after inoculation, and 5 died 1 to 8 days after onset. Filtered aqueous cat excreta were then used to induce diarrhea and death on 2 subsequent passages in cats. It is of interest that an uninoculated cat, living with those first inoculated, developed diarrhea and died. Excreta from this animal were used to initiate a second passage series. Filtered, twice diluted cat stool supernate from each series was fed in 3 ml and 5 ml amounts, respectively, to 2 human volunteers. This material, which in amounts of 1.5 ml and 1.7 ml produced a fatal disease in cats, caused no reaction in human subjects. The investigators noted that several cats had died in area of epidemic of human gastroenteritis under study, and acknowledged the possibility of "other incidental disease." However, they favored the idea that the same disease "prevailed among cats while it spread among men"(6).

The clinical description of the illness supposedly induced in cats by Yamamoto *et al.* (6) closely resembles that of infectious gastroenteritis of cats (feline distemper; feline panleucopenia). This disease has an incubation period of 6 to 14 days, and is characterized by depression, weakness, vomiting, profuse diarrhea, fever, and coma in 3 to 4 days (8,9). It is possible, therefore, that the Japanese investigators were dealing with a naturally occurring feline disease.

In the experiments here recorded 2 human gastroenteritis inocula of demonstrated infectiousness failed to produce disease in healthy cats.

Summary. Aliquots of supernates prepared from stools of volunteers with induced afebrile (Marcy) and febrile (FS) nonbacterial gastroenteritis and capable of inducing human disease were administered by stomach tube to cats. No recognizable disease was produced.

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Effect of Ultrasonics on Thromboplastinase-Labile Component and Toxicity of Injected Thromboplastin. (23055)

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The effects of heat on the thromboplastinase-labile component of tissue thromboplastins have been previously reported(1). Loss of thromboplastic potency due to heating of tissue suspensions and loss due to thromboplastinase are apparently distinct phenomena. Further, heat inactivated thromboplastin retained all of its *in vivo* toxicity for rabbits while thromboplastinase inactivated suspensions were non-toxic(2). Axelrod (3) recently reported on loss of potency of commercial thromboplastin following ultrasonic treatment. It was of interest to determine what effect ultrasonics would have both on the TPase-labile component of human brain thromboplastin and on the lethal effect upon intravenous injection of such thromboplastic suspensions into experimental animals.

Materials and methods. Thromboplastinase (TPase) was prepared as previously described(4). 2 mg crude TPase powder/ml water was employed as standard enzyme concentration. Heat inactivated enzyme (100° for 10 min) served as a control. *Thromboplastin (TP).* Thromboplastic suspensions were freshly prepared 1 hr before use by extracting 0.5 g acetone dehydrated human brain powder with 10 ml 0.9% saline at 50°C for 15 min and then centrifuging at 3000 rpm for 5 min. The supernatant was decanted and refrigerated prior to use. *Clotting time determinations (CT).* A modification(4) of the Quick one-stage test was used for assay of clotting potency. Duplicate determinations were made. *Thromboplastin dilution curves* were constructed as previously described(4). *Ultrasonic treatment.* Raytheon Model DF-101 200 watt sonic oscillator was used with a frequency of 10 kc. 50 ml samples of thromboplastic suspensions were treated at one time and aliquots were removed at intervals for assay of potency, preparation of dilution curves, treatment with TPase and injection into animals. *TPase assay.* The activity of TPase was followed by change in clotting potency and liberation of organic phosphorus after incubation at 37°C for 60 min(1). 10 ml of thromboplastic suspension and 1.0 ml TPase solution (2 mg) was used as a standard incubation mixture. *Experimental animals* employed were male albino rabbits weighing 2-2½ kg. Preparation of suspensions for intravenous injection and technics used have been described(2).

Results. The loss in clotting potency of human brain thromboplastic suspensions due to ultrasonic treatment is shown in Fig. 1. It can be seen that there was a progressive loss in potency over a time course. When dilution curves were plotted for aliquots removed at intervals (Fig. 2), it was evident that most of the activity had been destroyed quite rapidly. After 30 min of ultrasonic treatment clotting time was 19 sec. On the

TABLE I. Loss in Clotting Potency and Liberation of Organic Phosphorus Due to TPase Action on Ultrasonically Treated Thromboplastin.

Ultrasonic time (min.)	CT (sec.) after sonic treatment	CT (sec.) after TPase action for 60′ at 37°C	Phosphorus found (μg P/mg dry wt TP) Before TPase	After TPase
0	11.3	36.0	2.5	30.9
30	20.2	42.5	2.4	31.0
90	40.7	60.7	2.4	31.6

dilution curve of untreated thromboplastin, 19 sec represents approximately 5% thromboplastin. Similar calculations have been described previously(4).

Aliquots removed at 0, 30, and 90 min were subsequently treated with TPase. Table I summarizes the data obtained in one series of experiments on liberation of organic phosphorus after TPase action on these aliquots.

Although there was a definite loss in clotting potency due to ultrasonic treatment (11.3 to 40.7 sec), there was no destruction of the TPase-labile moiety of thromboplastin inasmuch as the same amount of organic phosphorus was liberated from each aliquot due to TPase action. The maximum phosphorus liberated by TPase in 180 min was 33.6 μg P/mg dry wt TP. Heat inactivated TPase caused no loss in clotting potency and no phosphorus was liberated.

These suspensions were then prepared for intravenous injection into rabbits in order to assay for lethal potency. Table II summarizes the findings. Loss of clotting potency due to ultrasonic treatment (11.3-40.7 sec)

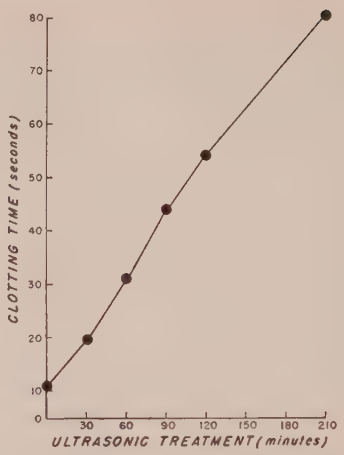

FIG. 1. Loss in clotting potency of human brain thromboplastin due to ultrasonic treatment.

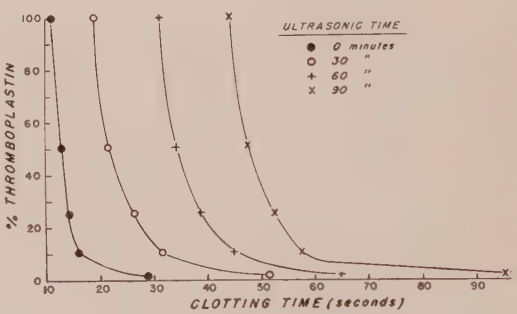

FIG. 2. Dilution curves of ultrasonically treated human brain thromboplastin.

did not result in a decrease in lethal potency of the thromboplastic suspensions. However, loss due to TPase action to approximately the same level (11.3-36.5 sec) resulted in a thromboplastic suspension no longer lethal in as high as 3x the normal lethal dose. Ultra-

TABLE II. Effects of Intravenous Injection of Variously Treated Brain Thromboplastic Suspensions in Rabbits.

Treatment	CT, sec.	No. animals	Dose, ml/kg body wt	Effects
None	11.3	4	1.5	4 died
Idem, with subsequent TPase treatment	36.5	4	1.5	4 no reaction
		2	3.0	2 *Idem*
		1	4.5	1 "
30′ at 10 kc	20.2	4	1.5	4 died
90′ " "	40.7	4	1.5	4 died
Idem, with subsequent TPase treatment	65.0	2	1.5	2 no reaction
		2	3.0	2 *Idem*
		1	4.5	1 "

sonically treated thromboplastin subsequently incubated with TPase lost its lethal potency for rabbits.

Discussion. The results of this study lend further support to the assumption(2) that TPase-labile moiety of thromboplastin and its *in vivo* toxicity are related. As in the case of heat inactivated thromboplastic suspensions, loss in clotting potency after ultrasonic treatment is not reflected by a concomitant loss in lethal potency for rabbits. In addition, the data indicate that there are at least 2 active thromboplastic moieties in tissue suspensions. By clotting measurements, the TPase-treated thromboplastin used in these studies was almost identical to that of the ultrasonically treated suspension yet the former was no longer toxic to rabbits while the latter had lost none of its toxicity. Quick (5,6) has presented evidence suggesting that brain thromboplastic extracts have a heat labile factor which acts directly with prothrombin, and a heat stable factor requiring a plasma factor to produce active thromboplastin. Whether the TPase labile factor is the same as, or distinct from, the heat stable factor is not known. However, the former appears to be important in producing the lethal effects seen upon injection of thromboplastin into experimental animals. In addition, it is apparent that clotting potency *per se*, as measured by *in vitro* clot acceleration, gives no indication of the physiological activity of tissue thromboplastins. The amount of phosphorus liberated by TPase under standard conditions appears to be a much better criterion.

Summary. The effects of ultrasonic treatment on human brain thromboplastic suspensions have been presented. Although causing a loss in clotting potency, ultrasonic treatment does not affect the toxicity of intravenous tissue thromboplastin nor has it destroyed the TPase-labile component. The results are discussed and possible implications presented.

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Method of Chorioallantoic Membrane Inoculation which Decreases Nonspecific Lesions. (23056)

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Since the original method of chorioallantoic membrane (CAM) inoculation was introduced by Goodpasture(1), numerous variations for preparing eggs for this route of inoculation have followed(2-5). The preparation of embryonated eggs for CAM inoculation with pock inducing viruses is of considerable importance if accurate counts of the pocks are to be made(5-7). Nonspecific lesions due to trauma invariably appear when eggs are prepared for CAM inoculation. They may be large enough to obscure the field to be counted or small enough to be mistaken for specific lesions. Their presence can be a source of variation in pock enumeration and differentiation and can influence the accuracy of pock titrations.

The purpose of this paper is to present a modification of the technic of CAM inoculation which greatly reduces nonspecific lesions. A statistical assessment of the accuracy of this method is reported.

Materials and methods. Virus and seed preparations. The virus used in this study

FIG. 1. Inoculation of CAM over rotated air space.

was the Yamada strain of variola virus obtained through the courtesy of Dr. Joseph E. Smadel, Walter Reed Army Institute for Research, Washington, D.C. It was isolated from a Japanese patient with a moderately severe case of smallpox and when received was in the second CAM passage. The virus was passed 3 times on the CAM of 11-12-day-old white leghorn eggs. On the sixth passage, eggs were inoculated with approximately 5000 infectious units per 0.05 ml and incubated at 35°C for 48 hours. A working pool of the virus was obtained by harvesting the infected membranes. A 20% suspension of harvested membranes in heart infusion broth, pH 7.0, was emulsified in a Waring blendor for 3 minutes and centrifuged for 10 minutes at 2000 rpm in an angle head centrifuge. The supernatant was stored in sealed ampoules in a dry ice chest at −70°C. Technic of preparing eggs for CAM inoculation. 1. Eggs were candled and an area of the CAM was selected which was well developed. Areas over large blood vessels were avoided. 2. Over the center of the air space a small hole was drilled through the shell with a Vibro-Tool* (V-3 hard tantalum carbide point). 3. A circle was outlined over the selected CAM area with the Vibro-Tool. The circle was drilled deep enough to loosen the flap of shell but not enough to penetrate the shell membrane. 4. The circular shell flap was detached from the shell membrane with a half spear point dissecting needle. 5. A drop of sterile saline was placed on the exposed shell membrane and a slit 1-2 mm long was made in the fibers of the membrane with a sterile dissecting needle. 6. After the saline had been in contact with the membrane for 1-2 minutes, the CAM was dropped by gentle suction with a rubber bulb thus creating an artificial air space. The suction was applied over the opening previously drilled in the air space. This was done in the beam of an egg candler so that the size of the air space could be visualized and controlled. The artificial air space was usually 20-25 mm in diameter. 7. The egg was rotated as soon as the artificial air space was formed. The newly formed air space was moved from the site of preparation to a well developed area of the CAM. This rotation was made to the left or right, depending on the location of the area of the CAM. The position of the artificial air space was then outlined. 8. A minute opening was drilled in the shell with the Vibro-Tool, over the area of the artificial air space. This opening was used for the introduction of inoculum. 9. The inoculum, 0.05 ml, was introduced into the egg (Fig. 1) from a 1 ml Luer-Lok tuberculin syringe fitted with a #26 or 27 gauge ¼ inch needle. The inoculated eggs were tilted slightly to distribute the inoculum evenly over the dropped area of the CAM. The eggs were then placed on their long axis in trays with the artificial air space uppermost and incubated undisturbed at 35°C for 72 hours. Leaving the openings in the shell unsealed in no way affected the viability of the egg or the formation of pocks. A few precautionary measures should be followed in this method. The slit in the shell membrane should be as small as possible, since the shell membrane is used as a natural seal when the eggs are rotated. In addition, in step 6, the artificial air space should be formed not later than 5 minutes after the application of the saline. It has been our experience that the exposed membranes dry rapidly. Undue delay makes the formation

* Burgess Vibrocrafters Inc., Grayslake, Ill.

TABLE I. Statistical Evaluation of 2 Technics of CAM Inoculation Employed for Calculation of Infectious Units of Variola Derived from Pock Counts.

	Non-rotated air space	Rotated air space
	Infectious units/ml ($\times 10^7$)	
	6.0	5.4
	3.6	7.6
	8.6	10.0
	9.4	11.0
	5.0	4.0
	4.8	9.2
	3.6	11.0
	5.0	8.6
	5.8	6.0
	5.0	6.2
	2.4	8.0
	2.2	6.2
	3.4	5.2
	1.4	5.2
	4.0	9.8
	6.0	6.8
	4.2	8.4
	2.8	7.2
	6.0	5.4
	4.8	11.2
	5.2	7.4
Mean	4.7×10^7	7.6×10^7
Stand. dev.	$\pm$ 1.93	$\pm$ 2.14
Stand. error of mean	$\pm$ 0.42	$\pm$ 0.46

of the artificial air space difficult and often causes it to be irregular in shape. Eggs were also prepared for CAM inoculation by the conventional method. In this procedure, the artificial air space was not rotated after the CAM was dropped. The inoculum was introduced onto the CAM through the exposed shell membrane. Virus titrations were carried out as follows: serial 10-fold dilutions ranging from 10^{-1} to 10^{-5} were made in heart infusion broth which contained 500 units of penicillin and 100 μg of streptomycin per ml. Virus dilutions 10^{-4} and 10^{-5} were inoculated on the CAM of 11-12-day-old embryonated eggs in quantities of 0.05 ml per egg by the methods described. Seven to 8 eggs were inoculated per dilution and incubated at 35°C. After 72 hours incubation, the CAM was removed from the eggs, washed and floated in Petri dishes containing formol saline. The pocks were counted with the aid of an illuminator. This instrument facilitates the examination of membranes by passing a reflected light across a Petri dish and illuminates the pocks by the darkfield principle. The number of infectious units per ml of the suspension was determined by counting the number of pocks per membrane and calculating for the dilution. Usually 7-8 membranes per dilution were counted. The average number of pocks per membrane was multiplied by 20 (for conversion to a ml basis) and by the dilution factor. The membranes employed to determine the concentration of infectious units were those which contained between 0 and 100 pocks.

Results. Accuracy of method. A total of 42 titrations were made to test the difference between the treatments employing rotated and non-rotated eggs. Twenty-one titrations were made with each treatment. One virus pool was used throughout the test and the dilution technics, inoculation, incubation and pock counting were kept standard.

Results of the treatments and statistical calculations are shown in Table I. Different mean values were apparent for both treatments, with a higher mean being obtained for the rotated method. A test of significance was made to evaluate the difference between the means of both treatments. The t value obtained was 4.580 for 40 degrees of freedom. The probability was less than 1 in 1000 that this value could occur by chance alone. The difference between the means was highly significant. The incidence of non-specific lesions in the non-rotated treatment was 66% higher than in the rotated treatment.

Discussion. The mean value found when the method of rotating the artificial air space was used was significantly higher than the value for the non-rotated method of CAM inoculation. The higher mean value obtained was probably due to the low incidence (4%) of nonspecific lesions. The non-rotated treatment induced a 70% incidence of nonspecific lesions which probably retarded or obscured specific pock lesions. Traumatic lesions generally occur in the area where the CAM is initially dropped. By rotation of the artificial air space to a new area of the CAM, the nonspecific lesions which may appear are left in the initial CAM area. Consequently, they do not interfere with pock formation or counting. A great deal of experience is not re-

quired to prepare a CAM area free of traumatic lesions with this method.

The principal advantage of this new method is that it reduces nonspecific lesions due to trauma, thereby increasing the accuracy of titrations. The reduction of nonspecific lesions also facilitates detection of pock inducing viruses in diagnostic work which may be masked by nonspecific lesions.

Summary. A modified method of CAM inoculation is described which greatly reduces nonspecific lesions and increases the accuracy of titrations.

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Effect of Estradiol on Fibrinolytic Activity of Rat Uterus.* (23057)

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The rat uterus contains an activator of plasminogen(1). The present investigation demonstrates a correlation between concentration of the tissue activator in the rat uterus and the estrous cycle.

Methods. Concentration of the tissue activator of plasminogen in the uterus was estimated by the quantitative and selective method described previously(2). In principle the activator is extracted with potassium thiocyanate solution, precipitated at acid reaction, redissolved and the concentration estimated by fibrin plate method(3). The activator concentration is calculated in units of a standard preparation/gram fresh tissue. The estradiol dipropionate solution contained 20 μg/ml in olive oil. White rats weighing 200-300 g were used. The weight of animals, macroscopic appearance and weight of the uterus and microscopic appearance of the vaginal smear were recorded. Concentration and amount of tissue activator in 3 series of animals (total 32) are presented in Table I. Six animals were killed immediately with chloroform and tissue activator concentration in the uterus estimated as described. Twenty-six animals were ovariectomized under ether anesthesia. Eleven of these, after 4-7 weeks without further treatment, were killed with chloroform and the uterus immediately removed and investigated. Eight of the ovariectomized animals were given 1 ml estradiol dipropionate solution intramuscularly every third day (from 5-11 times). Animals were killed in early estrus some days after the last injection, after examination of the vaginal smear (Allen-Doisy). The uterus was then removed and investigated. Seven of the ovariectomized animals were given 1 ml estradiol dipropionate solution intramuscularly every third day (from 2 to 4 times). The animals were then kept until vaginal smear showed them to be in late estrus (after 17 to 56 days). They were then killed and the uterus analysed. Additional confirmative control experiments are not included in the Table.

Results. The present investigation concerns tissue activator concentration in the rat uterus during the estrous cycle in the hope of confirming experimentally previous results with samples of human endometrium (in different stages of menstrual cycle) and human

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TABLE I. Effect of Estradiol on Content of Tissue Activator of Plasminogen in the Rat Uterus.

Treatment	Uterus wt, mg	Units/g & (avg)	Units/whole uterus & (avg)
Untreated controls	427	18 (16)	8 (5)
	325	16	5
	555	6	3
		9	
		18	
		27	
Ovariectomized 4 to 7 wk (no epithelial cells in vaginal smear) (anestrus)	58	17 (18)	1 (1)
	59	12	1
	55	12	1
	53	12	1
	95	14	1
	60	21	1
	90	23	2
	80	23	2
	100	23	2
	75	10	1
	105	29	3
Ovariectomized; estradioldipropionate 5 to 11 times; killed some days after last inj. (early estrus)	280	30 (20)	8 (7)
	276	29	8
	310	30	9
	400	30	12
	370	8	3
	380	15	6
	475	10	5
	475	8	4
Ovariectomized; estradioldipropionate 2 to 4 times; killed 17 to 56 days after last inj. (late estrus)	240	43 (53)	10 (15)
	250	56	14
	375	45	17
	280	56	16
	250	96	24
	260	34	9
	320	38	12

myometrium.

Fibrinolytic activity of human *endometrium* is caused by an activator of plasminogen(4,5) similar to the activator present in other human and animal tissues(6,7). Its concentration is increased in the secretory stage and decreases with age. High concentrations are also found in certain cases of pathological uterine bleedings. This suggests that the tissue activator in the endometrium is under hormonal influence and probably indicates a correlation with the occurrence of bleedings(4). Independent of age the human *myometrium* contains large amounts of an activator of plasminogen(8).

In the rat uterus the myometrium could not be separated from the epithelial layer. However, the epithelial layer in an uterus from a castrated animal is atrophic. Therefore all activity found in these uteri must be contained in the myometrium. The average concentration of the tissue activator/gram of uterine tissue in the ovariectomized animals (18 units), in uteri from the normal controls (16 units) and in animals in early estrus (20 units) is nearly equal. Similar results (not included in the Table) were obtained with 14 normal animals (not ovariectomized) which were killed from 1 to 6 days after receiving 5 to 7 injections of 1 ml estradiol dipropionate. Concentration in the uteri from these animals varied from 9 to 33 units/gram of fresh tissue (average 18). These results indicate that tissue activator concentration in the *myometrium* is independent of castration. Total amount of tissue activator in the uterus decreases after castration because the uterus decreases in weight. These results are in accordance with those previously obtained with the human myometrium(8).

The last 7 animals were killed when the number of epithelial cells in the vaginal smear began to decrease and were replaced by leucocytes (late estrus). Concentration of the tissue activator in this group was increased and ranged from 34 to 96 units/gram (average 53 units) corresponding to total amount ranging from 9 to 24 units (average 15). Although not proved it is most probable that increase in concentration from early to late estrus is caused by increase in concentration of the tissue activator in the epithelial layer. This increase in tissue activator concentration during late estrus, corresponds therefore to increased concentration in the human endometrium in the secretory stage.

The heart, brain and lung from 4 of animals in anestrus, 3 of those in early estrus and 2 in late estrus were also investigated to estimate concentration of tissue activator during different stages of the estrous cycle. Tissue activator concentration varied considerably from animal to animal, but no correlation to the estrous cycle was found.

Discussion. Page, Glendening and Parkinson(9) suggested a fibrinolytic enzyme to be present in the rabbit uterus. Our previous investigations have shown that fibrinolytic activity in uterus of rat and rabbit is caused by an activator of plasminogen and not by a fibrinolytic enzyme proper(1). The distinction between a fibrinolytic enzyme and

an activator of plasminogen is possible by comparing effects on normal bovine fibrin (which contains plasminogen) and heated bovine fibrin (with no plasminogen)(10). Extracts from the rat and rabbit uterus digested normal bovine fibrin whereas no digestion was obtained on heated fibrin.

The "enzyme" of Page *et al.* increased in concentration during estrogen withdrawal whereas castration caused a decrease. Contrary to these findings we found no decrease in tissue activator concentration after castration of rats. The discrepancy between our results on rats and those of Page *et al.* on rabbits could be caused by differences between the animal species.

Summary. 1. Concentration of tissue activator of plasminogen in the rat uterus has been estimated in different stages of the estrous cycle. 2. An increase in concentration was recorded during late estrus, whereas castration had no influence upon the concentration. 3. These results are in accordance with those found for human endometrium and myometrium.

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Metabolism of Esters by *Streptomyces nitrificans.** (23058)

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Considerable attention has been devoted to the inhibitive effects of physostigmine (eserine), prostigmine, miotine and other carbamates on acetylcholine esterase and cholinesterase(1,2), which are representative of the so-called "azolesterases"(3). Much less work has been done on effects of urethans on ordinary esterases, *i.e.* aliesterases, and on the enzymatic hydrolysis of carbamic acid esters(1,4). For example, eserine but not urethan inhibited a mycobacterial esterase that hydrolyzed tributyrin, ethyl and amyl butyrates, butyl propionate and benzoate, and ethyl valerate(5). In another study, 9 different urethans were attacked by a pig liver esterase extremely slowly if at all, but interfered with hydrolysis of methyl butyrate and tributyrin(6).

This difference in susceptibility of carbamic acid esters to pig liver esterase motivated the present investigations on metabolism of esters by *Streptomyces nitrificans,* an actinomycete which hydrolyzes(7,8), respires (9,10), nitrifies(8,9,11) and grows(7,10,12) on urethan and other carbamates. In an extensive survey of soil microflora, *S. nitrificans* was the only organism capable of developing in a mineral solution with ethyl carbamate as the source of available nitrogen, carbon, and energy(7). This unique culture was therefore assumed *a priori* to possess esterase activity not inhibited by urethan. The results presented in this paper show that urethan-grown cell material of *S. nitrificans* metabolizes a wide range of esters.

Materials and methods. Mycelium of *S. nitrificans* was grown on urethan in static cultures, harvested, washed, and homogenized as reported previously(10,12). Respiro-

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TABLE I. Metabolism of Esters by Urethan-Grown *S. nitrificans*.

Substrate, 10 μmoles/vessel	Interval,* min.	$Qo_2(N)$	% oxidation
None (autorespiration)	120	29	
Methyl formate	40	102	15
Ethyl "	50	116	17
Trimethyl orthoformate	40	266	22
Triethyl "	220	305	74
Ethyl acetate	30	169	21
n-Propyl "	70	170	19
n-Butyl "	80	162	17
Diethyl succinate	80	411	36
	190†	247	51
" malonate	110	242	34
" oxalate	60	247	24
Triethyl citrate	50	212	8
Dimethyl carbonate	40	313	44
Diethyl "	50	306	27
Ethylene "	40	311	52
Propylene "	40	285	30
Methyl cyanoacetate	110	168	64
Ethyl "	70	241	39
Methyl dichloroacetate	50	200	42
Ethyl nitrate	120‡	13	
Tetraethyl orthosilicate	40	250	9
Tributyl phosphate	120‡	44	
Benzyl cyanide	300‡	172	
Acetylcholine · HCl	300‡	6	
Choline · HCl	300‡	39	

* Unless otherwise indicated, intervals extend from time zero (substrate addition) to breaks in the curves.

† Diethyl succinate curve showed 2 breaks, one at 80 and a second after 190 min.

‡ No breaks in these curves.

metric experiments were carried out at 30°C in the conventional manner. Each Warburg vessel of approximately 16 ml capacity contained 0.1 ml of 50% KOH in the center well and 2.1 ml of fluid representing substrates and cell homogenate, equivalent to 1.4 mg cell nitrogen, in M/50 phosphate buffer supplemented with trace metals and adjusted to pH 7.0(10,12). All values for $Qo_2(N)$ have been corrected by subtracting the autorespiration.

Results. The data in Table I have been calculated from curves showing rates of gas consumption in Warburg respirometric experiments. The results reveal marked rises in oxygen uptake following addition of all substrates except ethyl nitrate, tributyl phosphate, acetylcholine, and choline. These 4 compounds caused some increases in gas consumption but to appreciably lesser extents. At the intervals indicated in Table I, rates of gas consumption decreased, *i.e.* the curves showed breaks, and tended to parallel the autorespiration except for the diethyl esters of succinate, malonate, and oxalate. With these 3 compounds, the rates of oxygen uptake, following breaks in the curves, continued to exceed that shown by the substrate-free vessel. For diethyl succinate a second break was observed after 190 minutes.

In other work reported elsewhere(8), an identically prepared cell homogenate which metabolized urethans under similar conditions was characterized by $Qo_2(N)$ values of 16, 35, 52, and 60 for 5.0 μmoles of methyl, ethyl, n-propyl, and n-butyl carbamates, respectively. Doubling the level at which these substrates were supplied did not significantly alter rate of oxygen uptake following their addition. Most esters listed in Table I were therefore metabolized from about 2 to 7 times as rapidly as the propyl and butyl urethans. The $Qo_2(N)$ for methyl formate was almost 6.5 times the corresponding value for methyl carbamate. The respective ethyl esters showed a 3.3-fold difference in $Qo_2(N)$. This variation in the reactivity for esters of carbamic acid as compared with other acids is probably not due to permeability difficulties since simple, low molecular weight, water-soluble esters generally traverse cell membranes with ease over a broad pH range(13). Furthermore, the mycelium of *S. nitrificans* used in these experiments had been homogenized by grinding(10,12).

When grown on peptone, glycerol, and yeast extract instead of urethan, the cell material of this organism showed no significant increase in oxygen uptake upon the addition of 10.0 μmoles of ethyl carbamate, for which the $Qo_2(N)$ was 0.06(10). In the present studies, however, such mycelium did show a rise in gas consumption when supplied with 10.0 μmoles each of triethyl orthoformate, $Qo_2(N) = 19$, and diethyl carbonate, $Qo_2(N) = 13$. But the corresponding values obtained with the urethan-grown preparation (Table I) were 16- and 26-fold greater. With ethyl carbamate as substrate, the $Qo_2(N) = 35$ for urethan-grown cell material(8) was 583 times the $Qo_2(N) = 0.06$ for mycelium cul-

tivated on peptone, glycerol, and yeast extract(10).

The relatively low activity of ethyl nitrate (Table I) is perplexing, since $Qo_2(N)$ values for 10.0 μmoles of ethanol alone or supplemented with 10.0 μmoles of $NaNO_3$ were 46 and 43, respectively. The increase in oxygen uptake associated with choline, $Qo_2(N) = 39$, was somewhat greater than for ethyl carbamate, $Qo_2(N) = 35$, but acetylcholine, $Qo_2(N) = 6$, showed the least effect of any ester. This reactivity of acetylcholine was of the same order of magnitude as the low acetylcholine-splitting ability observed with some bacteria(14,15). As regards choline, urethan caused a 67% inhibition in the activity of mature chicken bone marrow choline oxidase (16). But this avian tissue behaves differently from *S. nitrificans*. Urethan treatment did not reduce autorespiration or succinoxidase in chicken bone marrow, whereas both endogenous and succinate oxidation were significantly lowered by growing the actinomycete on ethyl carbamate as compared to peptone, glycerol, and yeast extract(10,12).

Of those esters which exhibited breaks in their curves, triethyl citrate and tetraethyl orthosilicate were associated with the lowest values for percentage of substrate oxidation, while triethyl orthoformate was respired to the greatest extent (Table I). In other studies with urethan-grown cell material, 17, 35, 36, and 18% of methyl, ethyl, n-propyl, and n-butyl carbamates were respectively oxidized(8). These data, however, are somewhat high since they were calculated on the assumption that the NH_2 group of the carbamate is completely converted to NH_3. Actually, some nitrite is formed(7,8,10), so that oxidation of the alcohol moiety of the esters does not account for all the oxygen consumed. This may explain at least in part the greater percentage oxidation of methyl and ethyl carbamates as compared to the corresponding formate esters. Since the urethans but not the formates can provide nitrogen for protein synthesis, one might have expected greater oxidative assimilation with the former.

Information on the metabolism of esters by actinomycetes is relatively limited. For *Streptomyces griseus* and other actinomycetes, tristearin, trilaurin, as well as vegetable and animal oils supported satisfactory growth and antibiotic production(17). Various glycolipids, phosphoglycerides, lipoproteins, cholesterol esters, acetylcholine and other esters present in myelin and human atherosclerotic aorta plaques served as growth or respiration substrates for many soil actinomycetes(18,19, 20). For a myelinolytic strain of *S. griseus*, acetylcholine allowed good cellular proliferation but increased rate of oxygen uptake only slightly in a Warburg experiment; choline, on the other hand, did not permit growth and depressed gas consumption(20). These studies and the present investigations are believed to provide the first information on acetylcholine esterase among actinomycetes.

Summary. A homogenate of urethan-grown *S. nitrificans* exhibited a marked increase in oxygen uptake when supplied with many esters. Carbamates generally exerted a lesser though significant stimulation of gas consumption. Growth on urethan therefore produced cell material containing esterase(s) characterized by broad spectrum activity. But whether carbamates are hydrolyzed by these or specific enzymes is not yet known. Acetylcholine showed the least activity of all substrates tested, although choline caused an appreciable increase in oxygen uptake.

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Milk Fat Synthesis from Acetate in Mammary Gland of the Cow. (23059)

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Previous publications from our laboratory have been concerned with the roles of short chain fatty acids, bicarbonate and glucose as precursors of milk-constituents(1-9). These have been studied by intravenous injection of various metabolites appropriately labeled with C^{14}. Acetate is the principal metabolite derived from fermentation of carbohydrates in the rumen(10), and we have shown that it is a precursor of all milk-constituents, as well as being extensively catabolised to meet energy requirements. As might be expected, plasma acetate is a potent precursor of the fatty acid moiety of milk fat, but acetate carbon is also utilized in synthesis of the glycerol moiety. In view of the large amount of acetate available to cow's tissues, this latter synthesis is probably an important source of glycerol. The evidence indicates that it takes place via the tricarboxylic acid cycle as well as via fixation of carbon dioxide derived from acetate oxidation(8,9).

The work of Popjak, Folley *et al.*(11,12) with perfused udders has shown that a considerable part of milk fat synthesis from small molecules takes place in the mammary gland itself. In this experiment 2-C^{14} labeled acetate was injected into the cistern of one quarter of the mammary gland of an intact cow, and radioactivities of milk fat components from that quarter were compared with those from the other 3 quarters and with those from a similarly labeled acetate injection made intravenously.

Methods. The technic for conducting tracer experiments with intact cows has been described by Kleiber(1). Following injection of labeled acetate, continuous samples of respired carbon dioxide were taken for the first 3 hours and then for short periods at intervals up to 35 hours. The cow was milked at 3, 10, 22, and 35 hours after injection. Details of injections and characteristics of the cows are tabulated in Table I. The fats were separated from milk samples and 4 crude fractions prepared from each. These were the glycerol, water-soluble steam-volatile fatty acids, water-insoluble steam-volatile fatty acids, and non-volatile fatty acids. Details of separation are described by Rogers (9). A sample of each fraction was combusted and the resultant carbon dioxide was trapped as barium carbonate. This was planchetted and counted at infinite thickness in a flow-gas counter. Udder injection of acetate was made into the right front quarter by passing a large blunt needle up the teat canal and depositing the acetate in the milk cistern.

TABLE I. Details of Animals Used and Isotope Injected.

	Udder inj. exp.	Intrav. inj. exp.
Wt of cow	478.5	470
Milk production, kg/day	7.4	7.6
Fat, %	4.8	5.7
Isotope inj.	1.20 mc $C^{14}H_3COOH$	3.86 mc $C^{14}H_3COOH$

TABLE II. Integrated Radioactivities of Milk Fat Constituents.

	$\sum_0^{35} \lambda_s \Delta t$				$\int_0^{35} \rho_s \, dt$
	Glycerol	Sol. VFA	Insol. VFA	NVFA	
Udder injection RFQ	30.51	249.6	431.04	171.78	3.96
" " 3Q	16.07	43.3	61.75	15.44	
Intrav. injection	27.9	79.7	83.1	16.8	62.78

This quarter was subsequently milked out separately from the others, and the data referring to this milk are henceforth indicated by the abbreviation RFQ. A similar abbreviation for the other 3 quarters is 3Q, and the intravenous injection data are indicated by the abbreviation i.v.

Results. The results are summarized in Table II and Fig. 1. Radioactivity of each sample was determined as "Standardized Specific Activity λ_s"(2) of which the units are μc/g atom carbon : μc injected/kg body weight. By dividing specific activity by administered dosage, it is possible to compare results from experiments in which different dosages were injected into animals of different weights. As metabolic processes take place at different rates, the specific activity of a milk-constituent is not necessarily a guide to the extent to which it is synthesized from injected metabolite. The best indication of the potency of an injected metabolite as pre-

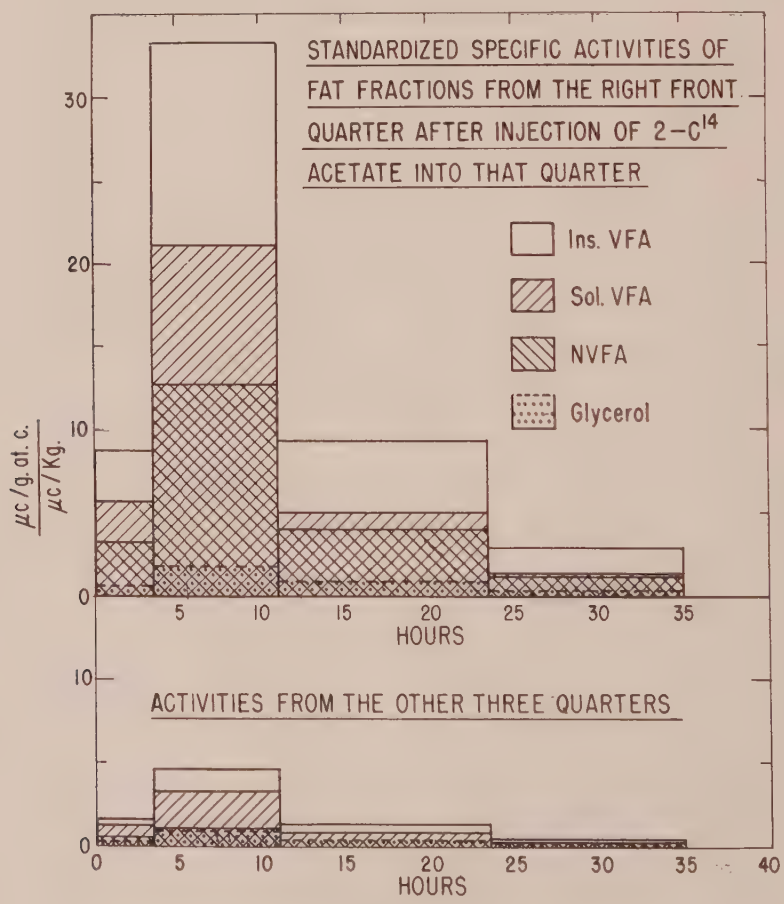

FIG. 1.

cursor of a milk-constituent is given by the time integral of specific activity of that constituent. This is easily determined since specific activity of a milk-constituent from any milk sample is the average specific activity of that constituent which has been secreted since the previous milking. Therefore, each average radioactivity can be multiplied by the number of hours in its milking period to give the integral for that period, and the sum of these will be the integral for the entire period $\sum_0^{35} \lambda_s \Delta t$. The time integral of the respired carbon dioxide activity is designated $\int_0^{35} \rho_s \, dt$.

The graphs in Fig. 1 illustrate the remarkably different activities of fat fractions from the RFQ and those from the 3Q. The fact that there is a difference shows that at least some of each fraction is synthesized in the mammary gland itself. The magnitudes of the differences are illuminating. Glycerol is only twice as radioactive from the RFQ as from the 3Q, whereas the 3 fatty acid fractions are more radioactive by factors of 6, 7, and 11 for soluble-volatile, insoluble-volatile and non-volatile fatty acids, respectively. In the RFQ the glycerol has much lower radioactivity than fatty acid fractions associated with it. In the 3Q, however, the relative difference is smaller and the ratio of radioactivities of glycerol and fatty acids is of a similar order to that seen previously where the labeled acetate was injected intravenously. From these 2 observations one can infer that there is less glycerol synthesis from acetate in the mammary gland than there is fatty acid synthesis. It is also apparent that mammary tissue cells are extremely active in synthesis of fatty acids of all chain lengths.

One of the most striking results from the udder injection experiment is that labeled acetate seemed to stay in the mammary gland and did not distribute throughout the body of the cow. This is clearly shown by the very small time integral of radioactivity of respired carbon dioxide, which is only 6.4% of that following a similar intravenous injection. It might be concluded from this that certainly less than 10% of injected acetate found its way into the circulation despite the vascularity of the udder. Similarly, the enormously greater radioactivity of milk fat constituents in the RFQ than in the 3Q and the persistence of the difference through 35 hours suggest that diffusion through the extravascular fluid is also limited. This apparent immobilization of injected acetate is not at all consistent with other observations concerning diffusion of small molecules in fluids of the body. An explanation is suggested by some other observations. As was previously reported(9), the most radioactive fat was recovered from the second milk sample after intravenous injection of a labeled precursor. The peak radioactivities of lactose and other milk constituents were seen in the first milk sample(2-8). The same phenomenon was observed even where labeled acetate was injected into the mammary gland, showing that the delay is not due to slow transport of fat to the udder from other synthetic sites. The work of Popjak, Folley *et al.*(11,12) with surviving slices of mammary tissue shows that actual synthesis of fatty acids from acetate is rapid. Furthermore, delay of peak radioactivity is observed in the glycerol moiety of fat as well, but peak of radioactivity in the lactose(8,13) is seen in the first milk sample in all trials, including this udder experiment. Since glycerol and lactose syntheses from acetate have common pathways, it is evident that the delay is not one of synthesis. Therefore, we have additional evidence for a previous suggestion(9) that the fat from the second milk sample is always more radioactive than the first because of a delay in actual secretion of fat.

If it is assumed, therefore, that injected acetate is rapidly utilized by cells adjacent to the milk cistern for fat synthesis and that this fat is only secreted several hours later, the apparent immobility of injected acetate seems less paradoxical. Continuance of the difference of the radioactivities of fat fractions between RFQ and 3Q even after 35 hours is understandable if the mammary gland is still secreting fat which was actually synthesized very shortly after injection of labeled acetate into the RFQ. The obviously small amount of injected acetate that diffused away from the RFQ can be accounted for in two ways. First, the rate of its incorporation

into fat molecules may have been much faster than the possible passive diffusion of acetate through the extravascular fluid. Second, the rate of acetate utilization for biosynthesis and catabolism in cells adjacent to the milk cistern might be so great that there is a steep diffusion gradient down towards that area, so that the number of acetate molecules moving in the opposite direction is small.

Summary. 1. 2-C^{14} labeled acetate was injected into the milk cistern of the right front quarter of a lactating cow. This quarter was subsequently milked separately from the other 3 quarters and radioactivities of milk fat constituents from each were determined. 2. The data confirm that there is a delay of several hours between synthesis and secretion of milk fat. 3. A surprisingly small amount of the injected acetate diffused into the other 3 quarters or into the rest of the body. This can be accounted for by its rapid utilization for milk synthesis near the site of injection. 4. Fatty acids of all chain lengths and glycerol seem to be synthesized from acetate in the mammary gland itself. The glycerol synthesis, however, is on a smaller scale.

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Further Studies of Prothrombin Derivatives.* (23060)

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Activation of purified prothrombin to thrombin can be achieved with the use of several combinations of materials separated from natural sources. All these "activator" factors are dispensable for even in 25% sodium citrate solution thrombin activity develops by autocatalytic mechanisms(1) thus indicating that the site on the prothrombin molecule which gives rise to thrombin activity is a structure arising only from changes in the prothrombin molecule itself. In addition to thrombin other derivatives can be obtained (2,3,4). Certain of these have a function in clotting of blood and it is likely that factor VII and plasma thromboplastin component (PTC) are derivatives of prothrombin(2,3). One derivative can again be reconverted to prothrombin by means of liver mitochondria preparations(5,6).

In the presence of thrombin, fibrinogen activates and subsequently polymerizes; and synthetic substrates, such as tosylarginine methyl ester (TAMe), are hydrolyzed(7). Thrombin can thus be regarded as an esterase, and in accordance with that view we have recently found that it hydrolyzes tributyrin quite effectively. Equally interesting is the discovery, reported here, that clotting activity of thrombin may decrease while the esterase activity is retained. Thus, we have

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TABLE I. Changes in Activity of Thrombin Preparation at 28°C.

Time, hr	Units thrombin activity*		
0	520 (520)	270 (270)	103 (103)
24	380 (515)	160 (280)	75 (100)
50	275 (500)	130 (280)	40 (100)
120	180 (510)	47 (270)	20 (80)
180	105 (510)	25 (150)	0 (40)

* Activity expressed in units/ml. Clotting activity is given first and esterase activity in parenthesis.

found another prothrombin derivative of interest in blood clotting and enzymology.

Methods. Purified biothrombin was prepared from purified bovine prothrombin as described by Seegers and Alkjaersig(8). The preparations were of high quality as indicated by several criteria. For example, the specific activity was equivalent to the highest previously obtained in this laboratory and in the ultracentrifuge virtually a single peak was observed. Clotting activity was measured by the method of Seegers and Smith(9), and esterase activity as described by Sherry, Troll and Wachman(7). For purposes of comparison a unit of clotting activity was regarded as equivalent to a unit of esterase activity.

Results. The biothrombin was placed in 0.9% NaCl solution and adjusted to pH 7.25. At room temperature clotting activity or capacity to activate fibrinogen was gradually lost (Table I). The esterase activity was more stable. Actually 3 different concentrations of the same thrombin preparation were studied. In the lowest concentration all clotting activity was lost while about 40% of the esterase activity remained. In the highest concentration studied, 80% of clotting activity was lost while all esterase properties remained stable. Experiments of this kind have been performed repeatedly and also with citrate thrombin. We find that it is possible to have all of the clotting properties disappear; however, the stability of the esterase is as yet not predictable. It is only possible to say that it is the more stable of the two activities.

Discussion. Many experiments have been performed to activate purified prothrombin so that another derivative besides thrombin is obtained(2,3,4). These derivatives evidently have physical-chemical properties slightly different from prothrombin itself(8) and all have the quality of being refractory to becoming thrombin in the presence of solutions wherein prothrombin ordinarily becomes thrombin. The view that some of these derivatives of prothrombin possess activities formerly attributed to "new" clotting factors, having no relationship to prothrombin, ascribes unusual qualities to a protein molecule. In furtherance of these ideas we suppose that the esterase and clotting activity are a property of a single molecule derived from prothrombin. This is biothrombin. By further modification of the molecule the clotting activity is lost, and only esterase activity remains. This we call *esterase thrombin.* We do not believe that these 2 kinds of activity are possessed by separate molecules derived from 2 kinds of precursors. It is interesting that we have not been able to have clotting activity without the esterase activity. We imagine that esterase thrombin can inactivate antithrombin and lyse fibrin clots in the same way as biothrombin can(10,11); and accordingly, are conducting experiments to see whether it does. Furthermore, an attempt is being made to activate prothrombin directly to esterase thrombin, for in the above experiments only the following sequence was observed: Prothrombin→Biothrombin→Esterase thrombin.

Summary. Purified bovine prothrombin preparations may lose the property of clotting fibrinogen but retain esterase activity. It is likely that a single molecule possesses the 2 activities. When biothrombin has lost clotting activity and only esterase activity remains, it is called *esterase thrombin.*

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Effect of Environmental Oxygen Tension on Intestinal Iron Absorption.* (23061)

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Recent reports by Reismann *et al.*(1) indicate that dissociable iron salts are readily absorbed through anatomically intact intestinal mucosa. Although the actual amount absorbed is small, toxic and lethal effects may result. Numerous studies have shown that intestinal absorption of iron is influenced by various types of anemia and fever(2). Granick(2) has postulated that oxygen content of the intestinal mucosal cells may influence absorption of iron by modification of the redox potential within the cell.

The purpose of this investigation was to study the relative amounts of ferrous iron absorbed at widely divergent oxygen tensions in the environmental air. It was desired to determine the effects of oxygen tension without accompanying anemia, infection, or modification of the diet.

Methods. Male and female albino rats, ranging in size from 150 g to 250 g were employed as test animals. All animals were starved for 24 hours prior to experimentation. Iron was administered in dose of 100 mg/kg of elemental iron as ferrous sulfate by stomach tube. Control serum iron levels were determined for each animal, following which the animals were fed the iron and immediately exposed to environmental oxygen tensions of 3 widely varying amounts. One group was exposed to oxygen tension of 54 mm Hg obtained by placing the animals at a simulated altitude of 26,000 feet. A second group remained at ambient oxygen pressure of 125 mm Hg (Denver altitude, 5,280 feet). A third group was exposed to pure oxygen at total pressure of 1390 mm Hg. The oxygen tensions and values employed are approximate, varying slightly with day to day barometric pressure and temperature. After 2 hours exposure to various oxygen tensions, the total serum iron was again determined. All serum iron values were obtained by modification of spectrophotometric method of Barkan and Walker(3) in which o-phenanthroline is used. Blood samples of 1.5 cc were drawn under light ether anesthesia from the right external jugular vein for serum iron determinations. A large number of control serum iron levels were made. One control group was exposed to 54 mm Hg oxygen and a second control group was placed in 1390 mm Hg oxygen for 2 hours without administration of iron. There was no visible necrosis or significant inflammation noted in the intestinal tract of animals autopsied at end of experimental periods.

Results. The results are tabulated in Table I. There was a significant increase in total serum iron levels in all 3 iron-fed groups. In addition there was a significant difference between serum iron values for each group of animals at each oxygen tension level. There was a small difference from control levels in animals exposed to high or low oxygen tensions, but not administered iron. A large group of control serum iron values were determined to establish the normal levels for the rat colony, including sex differences.

Discussion. From the observed results it

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TABLE I. Total Serum Iron Levels at Low, Ambient and High Oxygen Tension.

Groups	No. animals	Total serum iron, μg %: Normal	2 hr after varied O_2 tensions	% incr. over own controls	C.R.	C.R.*
Controls	85	182 ± 6‡				
Fe + low O_2 tension	15	210 ± 15	454 ± 38‡	+116	5.9	7.4 *
" + ambient "	17	177 ± 16	327 ± 24	+ 84.8	3.0	3.3 *
" + high "	16	158 ± 11	225 ± 19	+ 42.4	5.2	
No Fe, low O_2 tension	13		207 ± 30			.81†
" " high "	10		139 ± 33			1.28†

* C.R. $= \frac{D}{\alpha \text{ diff.}}$, compared to Fe + high O_2 tension. † Compared to normal controls.

‡ Stand. error.

appears that environmental oxygen tension does affect relative rate of ferrous iron absorption from the gastro-intestinal tract. Inasmuch as the non-beta-globulin bound iron is believed to leave the circulation rapidly(4), the serum iron level may not provide an accurate measurement of amount of iron absorbed. However, due to the short experimental period (2 hours) and the very small amount of iron absorbed at the dose level employed(1), it is felt that a reasonable estimation of relative rates of absorption was obtained. The effect of widely varied environmental oxygen tensions, as measured by serum iron levels, was rapid, being manifested within 2 hours. The mechanism by which low or high oxygen tension modifies iron absorption from the gastro-intestinal tract is unknown. Changes in liver and spleen iron content are known to occur at high altitudes (5). Hypoxia has been shown to increase succinic dehydrogenase levels(6). These phenomena are probably too slow in onset to explain the results of our experiments. Granick(2) hypothesizes that oxygen tension at the intestinal mucosal cell may affect passage of iron through the cell by modifying the redox potential within the cell. Low oxygen tension may retard oxidation of ferrous iron to ferric iron, and facilitate reduction of ferric iron to ferrous iron. Conversely, high oxygen tension could increase oxidation of ferrous iron to ferric iron. Such changes in oxidation-reduction potential within the mucosal cell, if they do occur, offer a possible explanation for increased iron absorption in presence of hypoxia, and a relative decrease in absorption during hyperoxia. The results obtained in these experiments indicate that iron absorption from the gastro-intestinal tract is a dynamic cellular process, and not a matter of simple diffusion.

Summary. 1. The effects of exposure of albino rats to 2 hours of low, high and ambient environmental oxygen tensions upon intestinal iron absorption were studied. 2. Total serum irons were increased 116%, 84.8% and 42.4% at environmental oxygen tensions of approximately 54, 125, and 1390 mm Hg respectively.

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A Hemagglutination Test for Detection of Adenovirus Antibodies. (23062)

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Adenoviruses have received considerable clinical and laboratory attention since their initial isolations by Hilleman(1) and Rowe *et al.*(2). The number of viruses in this group has grown to 14 as reported by Huebner *et al.*(3) and certain types have been associated with acute respiratory disease (ARD) in the civilian(4,5,6) and military (1,6,7,8) populations. The importance of adenovirus types 3, 4, and 7 in the military populations has been emphasized by development of adenovirus vaccines to reduce ARD in recruits(9,10). To justify the use of vaccines or to investigate the epidemiology of these agents in the military or civilian population the prevalence of adenovirus infection must be determined. This may be done by isolation of the agent, or by serological evidence relating to presence of this virus group since ARD may be influenced by a variety of organisms and conditions. Virus isolation and serological methods, employing neutralization and complement-fixation tests, have been used to determine incidence of adenovirus infection in military and civilian populations. These methods have been described in detail by Hilleman(7) and Rowe *et al.* (11). Many laboratories that lack isolation and tissue culture facilities must rely entirely on the complement-fixation test as a means of determining presence of adenovirus infection in a population. This test is limited because it is not type specific and previous infections with any one type many result in acute antibody titer level which may obscure a more recent infection with a new type.

To provide a simple laboratory *in vitro* test for diagnosis of adenovirus infection a modified hemagglutination test has been developed to detect antibody to adenoviruses. Although type specificity is not achieved, this test appears to be superior to the CF test as the titer rises are extremely high and elevated acute antibody levels are not found generally to interfere with the diagnostic interpretation.

Materials and methods. Hemagglutination (HA) test. Fresh sheep red blood cells (SRBC) suspended in Alsever's solution were washed 3 times with phosphate buffered saline at pH 7.2, and resuspended as a 4% packed cell concentration. Sensitization of the SRBC with dilute tannic acid was similar to the method of Boyden(12), as modified by Witebsky and Rose(13). An equal volume of tannic acid diluted 1:20,000 in buffered saline was added to the 4% suspension of SRBC. The mixture was held at room temperature (22-25°C) for 30 minutes. The cells were washed 3 times in buffered saline by centrifugation at 1500 rpm for 10 to 15 minutes and resuspended as 2% cell concentration by volume. Tannic acid sensitized SRBC were subject to slight hemolysis upon standing and were discarded 2 or 3 days after sensitization. An equal volume of adenovirus antigen diluted in buffered saline was added to the tannic acid sensitized SRBC and kept at room temperature for 30 minutes. The antigen treated cells then were washed in 1:200 normal rabbit serum by centrifugation at 1500 rpm for 15 minutes and resuspended in 1:200 normal rabbit serum at a 1% cell concentration. Acute and convalescent human sera were obtained from U.S. Naval recruits stationed at Great Lakes Naval Training Center. Acute and 21 day convalescent blood specimens were drawn from both afebrile, and febrile recruits who were hospitalized with upper respiratory illness. Zero and 21 day sera were drawn from recruits who had no recent history of acute upper respiratory infection and served as controls. All sera were inactivated at 56°C for 30 minutes before use. The dilution range was from 1:10 through 1:81,960. Occasionally, hemolysis of sensitized cells was observed in the 1:10 and 1:20 dilutions of sera. All dilutions were made in 0.2 ml volume of buffered saline with 0.2 ml

serological pipettes in 13 x 75 mm Kahn tubes rinsed in distilled water. In the final step 0.2 ml of adenovirus antigen treated SRBC was added to the diluted sera giving a final volume of 0.4 ml per tube. The tubes were kept at room temperature unless otherwise noted and read after 2 hours. *Antigen.* HeLa cells were grown in 1 l bottles containing 35 ml of Eagle's basal medium(14) with 10% human serum. After 3 to 5 days growth the cells were washed and the nutrient fluid replaced with Eagle's medium containing 3% horse serum. Each bottle was inoculated with 1 to 3 ml of stock adenovirus. The virus was harvested after all HeLa cells had fallen from the surface of the bottle, *i.e.* in 3 to 4 days incubation. The fluid was frozen and thawed 5 times and centrifuged at 2,000 rpm for 10 minutes. The supernatant which served as antigen was decanted and stored at −70°C. Serum titration endpoints were recorded as the reciprocal of highest serum dilution which produced a sedimentation pattern at 1+ hemagglutination according to the method of grading described by Stavitsky(15). Controls were carried out using the lowest serum dilution with normal SRBC and tannic acid treated SRBC without antigen. In addition, tannic acid treated cells with antigen were also employed as controls in each test. Unless otherwise noted, all hemagglutination tests were performed with adenovirus type 4 antigen. *Complement-fixation (CF) test.* The CF test was performed by making serial 2-fold dilutions of 0.2 ml aliquots of acute and convalescent sera. To these dilutions, 0.2 ml containing 2 units of adenovirus type 4 antigen was added followed by 0.2 ml containing two 75% hemolytic units of complement. The mixture was incubated at 37°C for 1 hour. Then 0.4 ml of sensitized SRBC was added, and the tubes were further incubated at 37°C for 30 minutes. The sensitized SRBC mixture consisted of equal volumes of 2% SRBC and 2 full units of amboceptor. Following this incubation all tubes were centrifuged at 1000 rpm for 1 minute and read for complement-fixation. Titration endpoints were determined as the reciprocal of highest serum dilution producing a 3+ fixation. All dilutions were carried out in physiological saline containing 0.01% $MgSO_4$ and 0.003% $CaCl_2$. The neutralization test was carried out in accordance with the method described by Rowe *et al.*(11) using procedure 2.

TABLE I. HA Antibody Titers with 2 Lots of Type 4 Antigens at Various Dilutions.

Antigen	Reference sera		Antigen dilution 1/4	1/8	1/16	1/32
Lot I	A	a*	640	160	40	<10
		c	40860	10280	2560	"
II		a	320	320	160	"
		c	81920	5120	1280	"
I	B	a	<10	<10	<10	"
		c	10240	2560	1280	"
II		a	<10	<10	<10	"
		c	10240	2560	1280	"

* a = acute serum; c = convalescent serum.

Results. To employ the proper dilution of antigen for the test, 2 adenovirus type 4 antigen lots were diluted and incubated with tannic acid treated cells for sensitization. After sensitization, the cells were incubated at room temperature with dilutions of acute and convalescent sera from 2 Naval recruits who had ARD and from whom type 4 adenoviruses were isolated. Table I shows the relation between antigen dilution and serum titer. The data indicate that higher antigen concentrations will result in higher titers in both the acute and convalescent sera of Sera A. It has also been observed that undiluted antigen produces high acute antibody levels which obscure convalescent titer rises in persons having adenovirus infections. By reducing the antigen concentration, it is possible to determine the antigen dilution which will detect acute antibodies in a reference serum (A in Table I) and which will show at least a 4 tube titer rise with the convalescent serum specimen. The antigen was similarly standardized in another dilution test with another reference serum (B, Table I) without acute antibodies. Using these 2 reference sera it is possible to determine the highest dilution of antigen which will react with 2 such reference sera in the above manner. This dilution is defined as one unit. In Table I the 1/16 antigen dilution is by definition one unit of antigen. In all subsequent tests

TABLE II. HA Antibody Titers to Heterologous and Homologous Adenovirus. HA antigens of recruits infected with adenovirus Type 4.

Patients		Antigen 1	2	3	4	5	7
NN	a*	80	40	<40	<40	40	80
	c	1280	80	2560	5120	>320	2560
PP	a	<40	320	<40	<40	<40	<40
	c	320	640	160	5120	"	80
QQ	a	<40	80	80	<40	160	<40
	c	"	"	640	1280	320	160
GG	a	"	320	<40	<40	<40	<40
	c	5120	2560	"	2560	"	"
JJ	a	160	<40	80	<40	80	320
	c	1280	"	640	640	40	1280
KK	a	160	320	80	<40	320	<40
	c	320	1280	40	2560	"	40
LL	a	80	320	<40	<40	80	<40
	c	320	1280	"	160	320	40
MM	a	<40	<40	160	<40	<40	<40
	c	40	"	320	1280	"	80
RR	a	<40	"	640	80	"	320
	c	"	"	1280	2560	"	"

* a = acute serum; c = convalescent serum.

one unit of antigen was used routinely. Pending further investigations on the stability of the HA antigen, it may be desirable to use 2 units of antigen in carrying out the test.

Specificity of the HA reaction was determined by titrating the sera of Naval recruits from whom adenovirus type 4 had been isolated with antigens of the other types. One unit of heterologous antigen was determined by antigen dilution and sera titration with sera A and B as reference standards and using this antigen dilution to determine heterologous antibody response. The data in Table II show homologous (Type 4) and heterologous reactions with acute and convalescent sera. It demonstrates that the HA test is non-specific since titer rises occurred to other antigens. Most of the cross reactions (4-fold rises) occurred with the type 7 antigen (67%), while types 1 (56%), 3 (45%) and 2 (33%) also showed crossing in a decreasing order. Type 5 demonstrated the least amount of crossing (22%) with sera of recruits who had been infected with type 4. These heterotypic crossings are somewhat in variance with the information obtained by Grayston *et al.* (16) which showed less crossing in the neutralization test with types 1, 5, and 7 than with types 2 and 3 in recruits infected with type 4 virus.

Stability of the antigen was determined by heating the type 4 (Lot II) preparation for 30 minutes at 56°C. The heated and unheated antigen was diluted to one unit, adsorbed to tannic acid treated cells and incubated at room temperature with serum dilutions (acute and convalescent) of recruits. Adenovirus type 4 was isolated from some of these hospitalized recruits. Heating the antigen to 56°C for 30 minutes does not appear to reduce antigenic activity of the preparation. The data in Table III show that heating the antigen increases some antibody titer endpoints, and, in addition, provides a means of inactivating live virus in the preparation (11).

Studies on effect of incubation temperature on hemagglutination reaction were carried out by incubating the sensitized cells and serum dilutions at 8°C, 25°C, and 35°C. The test was read after a 2 hour incubation. The results presented in Table IV show that the higher temperatures increase the serum titers. However, titer increases from 25°C to 35°C are not significant and 25°C was used routinely as a matter of convenience. Readings

TABLE III. HA Titers with One Unit of Type 4 Unheated Antigen and Heated Antigen.

Patient		Hospitalized	Virus isolation	Serum titer Unheated antigen	Heated antigen
C	a*	Yes	None	40	160
	c			81920	81920
D	a	"	Type 4	20	40
	c			5120	5120
E	a	"	"	80	20
	c			1280	2560
F	a	"	"	<10	<10
	c			160	1280
G	a	"	"	80	40
	c			2560	5120
H	a	"	"	20	<10
	c			40960	20480
I	a	No†	None	40	80
	c			"	40
J	a	"	"	"	"
	c			20	80
K	a	"	"	40	320
	c			"	"

* a = acute serum; c = convalescent serum.
† Men symptomatic and not hospitalized.

TABLE IV. HA Antibody Titers at Various Incubation Temperatures with Sera from Recruits with and without Adenovirus Isolations.

Patient		Type 4 virus isolate	Incubation temp. 8°C	25°C	35°C
1	a*	Yes	40	40	40
	c		640	1280	1280
2	a	"	40	40	40
	c		320	640	1280
3	a	"	80	80	40
	c		640	5120	10240
4	a	No	20	40	80
	c		40	"	40
5	a	"	10	20	"
	c		20	"	80

* a = acute serum; c = convalescent serum.

were also made at 5 hour and 18 hour intervals at room temperature and antibody titers were not significantly altered from the initial 2 hour observation.

Titrations were carried out for neutralization, CF and HA test with 13 pairs of acute and convalescent sera to compare titer levels and rises in both febrile (ARD) and afebrile Naval recruits. Neutralization and CF tests were performed with type 4 virus antigens since this agent was the only type isolated at the time these sera were collected. Results of the test comparisons are presented in Table V. The data suggest that the HA antibody is distinct from the CF or neutralizing antibody.

In asymptomatic cases (R, S and T) there was no CF or neutralizing antibody rise, nor was there an HA titer rise. Where a virus has been isolated, there was a neutralizing, HA and CF antibody rise in every instance. However, in cases where there is a neutralizing antibody rise without a CF rise, an HA antibody titer rise is demonstrated. This is shown with the sera of patients Y and Z. There was no corresponding rise in CF antibodies presumably due to presence of antibodies in the acute sera resulting from previous adenovirus infections. The HA test did demonstrate an antibody rise thus confirming the neutralizing antibody rise. If the CF test had been used, exclusively, these men would have been regarded as serologically negative. Conversely, patients U, V, W and X who had elevated acute CF antibodies showed no change in CF titer, and it would not have been possible to determine whether a present adenovirus infection had occurred. Since the HA test showed no titer rise, it may be presumed that these patients were not infected. This is confirmed by the neutralization tests.

It was of interest to compare time of appearance of CF antibodies and HA antibodies in recruits from whom adenoviruses type 4 and 7 had been isolated. Ten nasal washing specimens were obtained from these individuals at various times during their recruit training period. These samples were taken for adenovirus isolation in HeLa cells when the men became febrile (100°F or greater). The data in Table VI show the relation between virus isolation, CF and HA serology. There is a similarity in time of appearance of CF antibody and HA antibody which indicates that HA antibody may be produced simultaneously with CF antibody although the 2 antibodies appear to be quite different.

TABLE V. Neutralization, CF and HA Antibody Titers of Febrile and Afebrile Recruits.

Patient		Fever†	Virus isolation	Neutralization	CF	HA
N	a*	F	Type 4	<4	<4	<10
	c			64	128	320
O	a	F	"	4	<4	<10
	c			64	16	640
P	a	F	"	<4	<4	<10
	c			32	64	2560
Q	a	F	"	<4	16	<10
	c			64	256	640
R	a	AF	Negative	16	<4	2560
	c			"	"	1280
S	a	AF	"	<4	"	<10
	c			"	"	10
T	a	AF	"	"	"	<10
	c			"	"	"
U	a	AF	"	"	8	"
	c			"	16	"
V	a	AF	"	"	"	"
	c			"	"	"
W	a	AF	"	"	"	"
	c			4	"	"
X	a	AF	"	4	"	640
	c			4	"	"
Y	a	AF	"	<4	"	"
	c			8	"	2560
Z	a	AF	"	4	64	40
	c			16	128	640

* a = acute serum; c = convalescent serum.
† F = Temp. 100°F or greater; AF = Temp. less than 100°F.

TABLE VI. Virus Isolation, CF and HA Antibody Titers by Date in Naval Recruits.

Patient	Date (serum)	Date virus isolation	Titer CF	Titer HA
AA	1- 6		<4	<10
	1-17	1-16 negative	"	"
	2- 2	1-30 Type 4	32	1280
	2-18		128	5120
	3-10		256	2560
BB	1- 6		8	10
	1-27	1-18 Type 7	4	5
	3-10		16	80
CC	1- 6	1- 7 negative	<4	"
	1-27		"	"
	2- 5	2- 7 Type 7	4	160
	2-18		<4	"
	3-10		128	5120
DD	1- 6	1-24 Type 7	16	320
	1-27		"	2560
	2-18		64	10240
	3-10		"	"
EE	1- 6		"	640
	1-27	1-27 Type 4	32	"
	2-18		64	20240
	3-10		128	"
FF	1- 6	1- 5 negative	8	160
	1-27	1-18 "	4	"
	2-18	2- 3 Type 7	256	40960
	3-10		"	20480

Discussion and summary. The information presented shows that the HA test can be utilized for determining adenovirus antibody response. The test may be carried out with heated adenovirus antigen and yields results which relate to a current adenovirus infection. Hemagglutination antibody titrations can be performed at room temperature and readings may be made up to 18 hours after the initial 2 hour incubation without change in titration endpoints. Although the HA test does not achieve type specificity, this test appears to be more specific than the CF test. Huebner *et al.*(17) have reported 74%-90% heterotypic crossing in the CF test. The HA test reported here deals only with types 4 and 7 adenovirus infections and by extending these studies to include other adenovirus type infections it may be possible to utilize this test more specifically to determine infections to other types.

Based on preliminary data the HA test appears to be superior to the CF test in regard to determining infections in persons having CF antibodies in their acute serum due to previous adenovirus infections. The HA test and CF test show close correlation with respect to antibody titer rises and time of antibody appearance so that the HA test appears to be comparable to the CF test in its diagnostic value. Studies are in progress comparing HA, neutralization, and CF antibody titer responses in Naval recruits who have received the adenovirus vaccine.

Application of this HA technic to the immunology of other viruses should be considered. The ease of performing this test may provide a simple laboratory tool for determining serological responses to virus infections.

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Progesterone-Like Activity of a Series of 19-Nortestosterones. (23063)

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Hertz *et al.*(1) reported that 17α-ethynyl-19-nortestosterone is an orally effective progestational agent in rabbits, having an activity about 5 times that of 17α-ethynyltestosterone. Greenblatt(2) found the nor-compound was also effective in women, when administrated orally. Ferin(3) has recently reported that 17-methyl-19-nortestosterone has progestational properties in women, and Overbeek(4) found this compound to be active in rabbits. Pincus *et al.*(5) extended the series, observing that 17-ethyl-19-nortestosterone also has progestational effects in rabbits.

In this laboratory, extensive studies on the biological properties of 19-nortestosterones carried out over the past several years, indicated a marked progestational activity of these compounds (Drill and Saunders).† The present paper deals with the progestational effects of a series of 19-nortestosterones in which the 17α side chain ranges from H to C_8H_{17}, with both saturated and unsaturated linkages.

Methods. White rabbits weighing about 2 kg were ovariectomized. After a 2 week recovery period, they were injected subcutaneously, daily for 6 days, with 5 μg estrone. On the day following the last injection the abdomen was opened and a 1-inch segment of each uterine horn was isolated after the method of McGinty *et al.*(6). The test substance, in 0.1 ml corn oil, was injected into one uterine horn while a like amount of corn oil was injected into the opposite horn as a control. Three days later, the animals were sacrificed, cross-sections of the uteri were fixed in Zenker's and prepared for histology. The degree of glandular proliferation was graded from +1 to +4. Additional studies were made, using intact, immature rabbits which had been primed with 6 daily injections of 5 μg estradiol each. They were then treated orally or subcutaneously with the test compound in oil, daily for 5 days. On the day after the last injection the animals were sacrificed and the uteri were studied histologically.

Results. Intrauterine assay. The average responses to the various doses are shown in Figs. 1-3. The ordinates are arbitrary scales on which 1 denotes that the endometrial glands are only small straight indentations, similar to those usually found in the estrogen-primed controls. Further development with slight branching of a few glands is designated 2, while 3 indicates that there are many branched glands. Grade 4 is reserved for those uteri which show so much branching that, in cross-section, many portions appear detached. The number of animals at each dose is also indicated. The average rating for 545 control, estrogen-primed uteri was 1.04. Only 2 of these control uteri had ratings of 3 and 4 respectively. In both of these cases the contralateral horn had been injected with a high dose of an active compound. A systemic effect, or leakage, may account for these false positives.

The activity of progesterone is shown in Fig. 1. It will be seen that a dose of 0.5 μg is adequate to produce a definite response, with an average value of 2.3. The data for

† Drill, V. A., and Saunders, F. J., *Proc. of a Conference on Anabolic Agents.* G. D. Searle & Co., Chicago, Apr. 9, 1956.

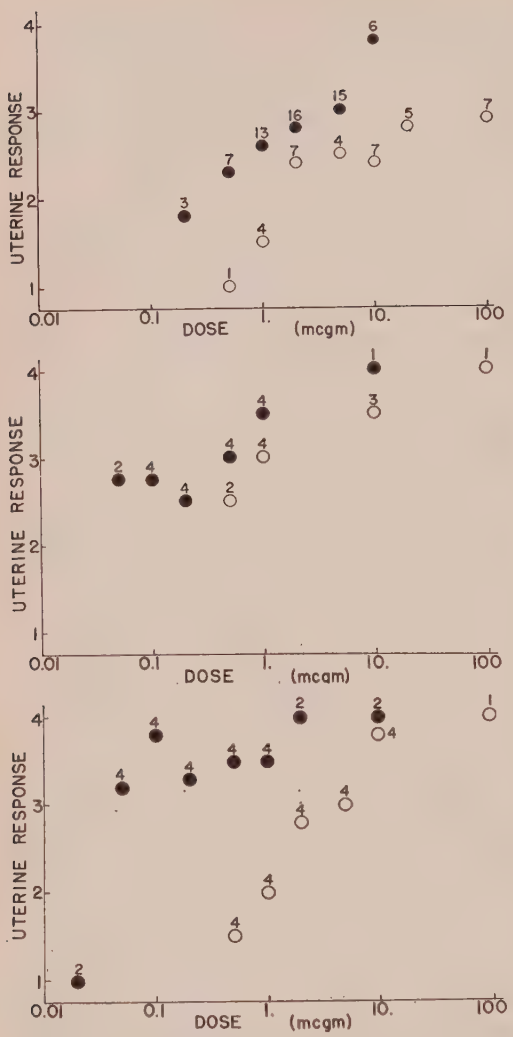

FIG. 1 (top). Responses of endometrial glands to local applications of progesterone (●) and 17-ethyl-19-nortestosterone (○). No. above points indicate No. of rabbits used.

FIG. 2 (middle). Responses of endometrial glands to local applications of 17-propyl-19-nortestosterone (●) and 17-octyl-19-nortestosterone (○). No. above points indicate No. of rabbits used.

FIG. 3 (bottom). Responses of endometrial glands to local applications of 17-butenyl-19-nortestosterone (●) and 17-allyl-19-nortestosterone (○). No. above points indicate No. of rabbits used.

19-nortestosterone and 17-methyl-19-nortestosterone are not presented, but in both cases, doses up to 100 μg were inactive, with average responses less than 1.5. On the other hand, 17-ethyl-19-nortestosterone gave responses indicating that this compound is about ¼ as active as progesterone in this assay. As the side-chain was lengthened, the activity was increased further, the propyl compound being about 5 times as active as progesterone (Fig. 2). However the octyl compound appeared to be somewhat less active than the propyl.

At least with the lower members of the series, unsaturated linkages in the side-chain appeared to decrease activity. The 17-vinyl and 17-ethynyl compounds were both inactive in this assay at doses up to 100 μg. The allyl compound was about ⅕ as active as the propyl (Fig. 3). The 17-butenyl compound is the most active tested to date (Fig. 3) but in preliminary tests the butyl compound appears to be nearly as potent. In the case of the butyl compound, the side-chain is straight; in the butenyl it appears to be branched.

17-Propyl-4,5-dihydro-19-nortestosterone and 11β-hydroxy-17-ethyl-19-nortestosterone were also found to be inactive at doses of 100 μg.

Systemic assay. Administered subcutaneously in the estrogen-primed immature rabbit (Clauberg Assay) a different order of activity was found. The minimal effective daily dose of progesterone was found to be 0.05 mg. 19-Nortestosterone was ineffective at 10 × this does but 17-methyl-19-nortestosterone was more active than progesterone. The 17-ethyl compound appeared to be 5 × as potent as progesterone. This value agrees with findings of Pincus *et al.*(5). However the butenyl compound, though more active than progesterone itself, was less active than 17-ethyl-19-nortestosterone. Table I shows the average ratings obtained with these compounds at the several doses, as well as the number of animals used.

Discussion. The group of nortestosterones studied here represents a wide range of biological activity. The androgenic and anabolic activities of some of this series have been reported(7). These data have been included in Table II to show the lack of correlation between the progestational response and androgenic or anabolic activity. While ana-

TABLE I. Endometrial Response in Estrogen-Primed Immature Rabbit after Subcutaneous Administration of Various Doses of Several Steroids (Clauberg Assay).

Compound	Daily dose, mg, subcut. 1.	.5	.2	.1	.05	.02	.01	.005
Progesterone	3.9 (4)	3.8 (2)	3.4 (4)	3.1 (7)	2.9 (8)	1.1 (8)		
19-nortestosterone	1.5 (4)	1.0 (1)	1.0 (2)					
17-methyl-19-nortestosterone	3.5 (2)	3.3 (4)	2.7 (6)	2.8 (6)	2.7 (6)	2.4 (6)	2.0 (3)	
17-ethyl-19- "	4.0 (1)	3.5 (2)	3.0 (3)	3.5 (4)	3.0 (6)	3.4 (5)	3.1 (6)	1.8(4)
17-vinyl-19- "	3.5 (2)			3.5 (2)				
17-allyl-19- "				3.3 (4)			3.0 (3)	
17-butenyl-19- "	3.3 (3)		3.9 (4)		3.7 (4)	3.3 (6)	2.2 (6)	1.0(4)
17-octyl-19- "			1.0 (4)	1.0 (4)			1.0 (4)	

Endometrial responses were graded from +1 to +4 (see text). When the avg response for 4 or more rabbits is above 2, the compound is considered active. No. in parentheses indicates No. of rabbits at that dose.

bolic and androgenic potencies were greatest among the lower members of the series, progestational effects were most marked in the higher members. In the saturated series, the methyl and ethyl derivatives as well as nortestosterone itself all have equivalent androgenic and anabolic potencies. However progestational effects increased markedly as the 17-side-chain was lengthened. This was especially noticeable in the intrauterine assay, where propyl and butyl compounds were found to be 5 times as active as progesterone and even the octyl compound showed marked activity.

TABLE II. Relative Activities of a Series of 19-Nortestosterones Compared to Several Standards.

Compound	Progestational potency Systemic	Intrauterine	Androgenic potency	Anabolic potency
Progesterone	100%	100%	<2	<5
Testosterone propionate		<1	100%	100%
19-nortestosterone	<10	"	6	100
19-nortest'ter'ne derivatives				
17-methyl-	250	<1	6	100
17-ethyl-	500	25	6	"
11β-hydroxy-17-ethyl-		<1	<2	<10
17-vinyl-	>50*	"	2	20
17-ethynyl-		"	estrogenic	1
17-propyl-	200*	500	<5	10
17-propyl-4,5-dihydro-	<5	<1	<2	<5
17-allyl-	500*	100	2	10
17-butyl-		500*	<2	<5
17-butenyl-	250	1000	2	5
17-octyl-	<25	100		

* Preliminary data.

The introduction of a double bond in the 2 carbon side-chain markedly reduced anabolic and androgenic activity. With a triple bond, these activities were further reduced and in fact the ethynyl compound shows some estrogenic effects. In both cases, progestational effects, as determined by intrauterine assay, were abolished although both compounds show endometrial gland proliferation when administered systemically. In the propyl series, introduction of a double bond in the side-chain (allyl) had little effect on androgenic or anabolic properties. As was the case with the 2 carbon side-chain, however, intrauterine assay showed a marked reduction in progestational activity. Administered systemically, both propyl and allyl compounds were significantly more potent than progesterone. Saturation of the 4-5 double bond of the propyl compound not only abolished its androgenic, anabolic and progestational effects but imparted to this compound anti-hypertensive properties.*

Several of the compounds were further tested for their ability to maintain pregnancy in ovariectomized rabbits. Does were bred and 10 to 14 days later they were ovariectomized. On the day of operation and continuing for 6 or 7 days they received 1 mg per kg of the various compounds daily, subcutaneously. They were sacrificed on the day after the last injection. All 9 control rabbits showed complete resorption of the fetuses with no apparent development of the fetus

* Data to be published by F. M. Sturtevant of this Division, 1957.

after spaying. On the other hand 14 of 15 rabbits treated with progesterone had 1 or more normal living fetuses at time of sacrifice. None of 5 rabbits treated with 19-nortestosterone showed any protection. With 17-methyl-19-nortestosterone 1 rabbit had living fetuses and the other 4 had some further placental development although no fetuses remained. With 17-ethyl-19-nortestosterone, 9 had one or more apparently normal fetuses. Two more showed some further development after spaying but the young were resorbing at the time of sacrifice. Three additional rabbits showed no protection. With 17-propyl-19-nortestosterone, 4 of the 5 rabbits showed normal development while the fifth showed decided fetal growth, although the young died before autopsy. The higher analogs have not yet been tested.

It has been pointed out that, compared to progesterone, some of these compounds are much more effective when administered systemically rather than directly into the uterus. Preliminary data indicate that the oral activity of this series, in general, is very marked with a high oral/parenteral ratio. These findings suggest that these norsteroids may be metabolized into more active compounds. Further studies on this series are in progress.

Summary. 1. Progestational activity of a series of 17-substituted 19-nortestosterone derivatives was investigated using both intrauterine and systemic assays in rabbits. Several of the substances studied possessed a high order of progestational activity. This activity was not related to either androgenic or anabolic potency of these compounds. Of the compounds studied 17-butenyl appeared to be the most potent, possessing about 10 times and 2½ times the activity of progesterone in intrauterine and systemic assays, respectively. 2. Progesterone-like activity of these 19-nortestosterones was further demonstrated by the ability of some members of the series to maintain pregnancy in ovariectomized rabbits.

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Effects of Aldosterone and Desoxycorticosterone on Tissue Electrolytes.* (23064)

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Desoxycorticosterone (DOC) has been used in replacement therapy for adrenal insufficiency for more than 20 years. A major portion of research on the salt-retaining function of the adrenal cortex has consisted in a comparison of the effects of adrenalectomy and administration of desoxycorticosterone acetate (DCA). The recent research on aldosterone, the natural hormone whose actions DOC mimics, has revealed not only quantitative but also qualitative differences between the 2 steroids(1). Inasmuch as the major biological role of these compounds probably is to modify electrolyte transport, it seemed of interest to compare the effects of the 2 steroids on cellular ion transport systems. For this purpose, the effects of administra-

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TABLE I. Effect of DCA and Aldosterone on Total Electrolytes and Water in Plasma, Brain, and Muscle of Mice.*

	Plasma				Brain				Muscle			
	H_2O, %	Na	K	Cl	H_2O, %	Na	K	Cl	H_2O, %	Na	K	Cl
		mEq/l				mEq/kg wet tissue				mEq/kg wet tissue		
Controls	93.13 ±.25	159.0 ±3.8	3.83 ±.2	123.4 ±.9	79.76 ±.55	42.43 ±.25	105.5 ±.8	28.69 ±1.40	76.44 ±.11	23.88 ±.04	108.7 ±.3	15.84 ±.33
DCA	93.12 ±.01	163.2 ±1.8	3.37 ±.07	122.5 ±.2	80.34 ±.03	44.65 ±.14	109.2 ±.1	29.40 ±.12	76.61 ±.13	27.46 ±1.28	109.6 ±3.3	14.93 ±.12
Aldosterone	92.71 ±.34	169.8 ±5.2	3.71 ±.25	125.3 ±1.3	79.88 ±.59	44.17 ±.90	107.3 ±.8	30.00 ±.16	76.74 ±.03	26.07 ±.44	113.8 ±2.7	18.39 ±1.95

* Values given are mean ± stand. error.

TABLE II. Effect of DCA and Aldosterone on Extracellular and Intracellular Distribution of Electrolytes and Water in Brain and Muscle of Mice.*

	Cl space, %		Intracellular concentration: H_2O, %		Na, mEq/kg cell water		K, mEq/kg cell water		Extracell. Na / Intracell. Na		Intracell. K / Extracell. K	
	Brain	Muscle	Brain	Muscle	Brain	Musc.	Brain	Muscle	Brain	Muscle	Brain	Muscle
Controls	20.59 ±1.22	11.40 ±.30	59.17 ±1.75	65.04 ±1.96	15.0 ±4.5	8.2 ±1.4	177.0 ±3.9	166.5 ±.3	12.0 ±3.9	20.4 ±3.8	45.6 ±3.6	42.8 ±2.5
DCA	21.22 ±.08	10.80 ±.09	59.12 ±.05	65.81 ±.04	15.7 ±.7	14.3 ±2.4	183.4 ±.1	166.1 ±5.1	10.7 ±.6	12.0 ±2.1	53.5 ±1.0	48.5 ±.5
Aldosterone	21.09 ±.24	12.92 ±1.16	58.79 ±.84	63.82 ±1.13	12.7 ±.2	5.49 ±3.6	181.3 ±1.3	177.5 ±7.0	13.7 ±.7	56.0 ±37.4	47.9 ±3.7	46.8 ±1.4

* Values given are mean ± stand. error.

tion of aldosterone and DCA on electrolyte composition of plasma, brain, and muscle were determined.

Methods. Thirty-six mice (Carworth Farm, CF #1 strain) weighing between 35 and 45 g were divided into 3 approximately equal groups. The groups consisted of (1) controls, (2) animals given DCA, and (3) animals given aldosterone. All groups were injected by the subcutaneous route once daily for a period of 4 days; the control group received 0.1 ml of isotonic sodium chloride solution containing "Tween 80," the DCA group received 0.5 mg of DCA suspended in the same vehicle, and the aldosterone group‡ received 20 μg of aldosterone dissolved in 0.1 ml of isotonic sodium chloride solution for 3 days and 8 μg of aldosterone on the last day. Sixteen hours after their terminal injection, the mice were anesthetized with 2.4 mg pentobarbital sodium (about 60 mg/kg), blood was drawn from the inferior vena cava into heparinized syringes and immediately centrifuged, and cerebral cortex and hind leg muscle were collected in tared bottles after blotting to remove superficial blood. Electrolyte determinations were performed on pooled samples comprising the plasma or tissue from 4 mice. The content of water, sodium, potassium, and chloride was determined in plasma, brain, and muscle samples according to methods previously described(2). Intracellular water and electrolyte concentrations were calculated by the method of Hastings and Eichelberger(3).

Results. The data obtained are summarized in Table I, which presents the analytical results for total plasma and tissue electrolytes, and Table II, which presents the calculated intracellular values. The administration of DCA resulted in an elevation of Na concentration and a lowering of K concentration in plasma, chloride concentration was unchanged. In brain, chloride space, which is assumed to measure extracellular

‡ The authors wish to express their appreciation to Dr. R. E. Knauff, Upjohn Co. for the gift of aldosterone.

volume, was unchanged. The concentration ratio of brain Na $\left(\frac{Na_e}{Na_c}\right)$ did not change significantly, whereas the concentration ratio of brain K $\left(\frac{K_c}{K_e}\right)$ was markedly elevated. In muscle, $\frac{K_c}{K_e}$ was elevated, despite a reduction of $\frac{Na_e}{Na_c}$. The reduction in the concentration ratio of muscle Na was related to a significant elevation of intracellular Na concentration.

Aldosterone elevated the plasma Na concentration markedly and lowered the plasma K concentration slightly. In brain, aldosterone did not affect the chloride space; it lowered intracellular Na concentration and slightly raised $\frac{Na_e}{Na_c}$ and $\frac{K_c}{K_e}$. In muscle, aldosterone definitely lowered intracellular Na and markedly increased $\frac{Na_e}{Na_c}$ ratio, and K accumulation occurred, although only to a moderate degree.

Discussion. The important effects of the 2 steroids on cellular electrolytes may be stated briefly. DCA elevated the K ratio without changing the Na ratio significantly in brain, whereas in muscle, the K ratio was elevated only slightly and the Na ratio was reduced. In contrast, aldosterone elevated K and Na ratios in both brain and muscle. The absence of an effect of DCA on brain Na differs from previously reported findings and may be presumed to be related to the short period of treatment and the low dose used. In sufficient dosage, DCA produces a definite decrease in intracellular Na and an increase in the Na ratio in brain(4,5).

The unequal partition of electrolytes across nerve and muscle membranes is consequent to active extrusion of Na from cellular fluid (6,7). Further, evidence is accumulating which indicates that this extrusion is not a simple ion pump, but that it is linked in some way to K influx. Thus, both in frog muscle and invertebrate nerve, it has been shown that Na efflux is reduced in the absence of external K(8,9). More indirect evidence for such a coupled system has been obtained in mammalian spinal motoneurons(10) and in cerebral cortex.§ Accordingly, it would be predicted that the administration of an agent that enhances ion transport would cause increases in $\frac{Na_e}{Na_c}$ and $\frac{K_c}{K_e}$ in both brain and muscle.

It is evident that aldosterone fulfills these predictions and there seems to be no difficulty in accepting the role of this hormone as a substance that promotes ion transport. In the case of DOC, although the effects on brain fit into the general hypothesis, those on muscle do not. The paradoxical finding that the administration of DCA causes a decrease in $\frac{Na_e}{Na_c}$ and a relatively minor effect on $\frac{K_c}{K_e}$ in muscle has been noted by previous authors (11,12) and is corroborated by the results presented here.

Either of 2 proposed mechanisms could be advanced to explain the effect of DOC on muscle electrolytes. It is possible that DOC simply acts differently on muscle than on brain and that its actions on muscle cause a reduction of electrolyte transport. It is also possible that the synthetic steroid is sufficiently different chemically from aldosterone that, although it mimics the hormone at some sites, it has no direct action on muscle. If this is the case, the variations in electrolyte composition which are observed after DCA administration must be secondary to its effects at other sites. Thus, the administration of DCA causes a loss of K from the body through both the kidney and the gut(12-14) and, as a result, plasma K concentration falls. Inasmuch as the membrane potential determines $\frac{K_c}{K_e}$ rather than the cellular K concentration and inasmuch as Plasma K concentration falls, K would be expected to shift from muscle cells into the extracellular fluid. The K ratio would either remain constant or be elevated slightly even though K has been lost

§ Unpublished observations.

from muscle. In association with this loss of K, muscle cells would gain Na, as demonstrated by the work of Heppel(15,16). This hypothesis, that the changes in muscle electrolytes observed after the administration of DCA result from an indirectly induced K loss and not from a direct effect of the steroid on the muscle membrane, could be tested in several ways. An indirect method would be the examination of the changes in muscle electrolytes induced by aldosterone and DCA in animals in which plasma K concentration is not allowed to fall. The observations of Ferrebee *et al.*(11) indicate that the administration of K salts prevents DCA-induced Na accumulation in muscle and may be taken as supportive evidence for the second hypothesis. A more direct test would be the measurement of muscle membrane potentials after treatment with each of the two steroids. In this connection, preliminary findings of J. W. Woodbury and Koch§ show that the resting potential of isolated frog sartorius muscle is unchanged by immersion in Ringer's solution saturated with DCA.

Thus, several independent lines of evidence suggest that DOC is devoid of direct effect on muscle and that the changes in muscle electrolyte composition which are observed are the passive result of actions of the steroid at other sites. In contrast to this, aldosterone appears to be capable of enhancing electrolyte transport at all sites which have been studied.

Summary. Plasma, brain, and muscle electrolytes were determined in mice given DCA and aldosterone. DCA increased the concentration ratio of K $\left(\frac{K_c}{K_e}\right)$ without affecting the ratio of Na $\left(\frac{Na_c}{Na_e}\right)$ in brain; it increased the concentration ratio of K moderately in muscle and decreased the concentration ratio of Na. Aldosterone increased concentration ratios of Na and K in both brain and muscle. An explanation of this difference in the effect of the two steroids on skeletal muscle electrolyte is proposed.

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Enhancement of Pathogenicity of Enterobacteriaceae for Mice by Wetting Agents. (23065)

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The enhancement of pathogenicity of many bacteria under circumstances which have suggested an increase of invasiveness is well known. Typical instances are those of spreading factors discovered by Duran-Reynals(1) and of mucin, as demonstrated by Nungester *et al.*(2), Miller(3,4), and Rake(5,6). While the mode of action of the latter remains controversial(7-11), spreading factors have been identified with hyaluronidase(12,13) and their effect is ascribed to an increase in tissue permeability which results from enzymatic hydrolysis of hyaluronic acid and facilitates penetration and scattering of infectious organisms.

The assumption that wetting agents, which promote closer interfacial contacts, might facilitate access of microorganisms to the intimacy of the host's tissues through liquid or semiliquid media led the author to investigate the effect of surface tension depressants on bacterial infection. In a preliminary investigation(14) the effect of dioctyl sodium sulfosuccinate (Deceresol-OT*) on pathogenicity of *Salmonella typhosa* was determined for mice inoculated intraperitoneally. The innocuousness of this wetting agent having been established for the bacteria and inoculated animals, it was found that Deceresol-OT in 5 tests consistently increased pathogenicity of the organism, as shown by LD_{50}'s 100 to 2,000 times smaller than in the controls. On the basis of this finding, the investigation was extended to include other bacterial species and another wetting agent. The results reported here suggest a possible association between the property of decreasing surface tension and the power to enhance bacterial pathogenicity.

Method. Wetting agents employed were Deceresol-OT and sodium lauryl sulfate (Duponol W.A.). The former is soluble in water at the rate of 1.5 g/l at 20°C and 2.3 g/l at 40°C, soaking of several hours being necessary to dissolve it; the pH of the solution varies from 6.5 to 7.0, and at concentration of 1:1,000 surface tension of water is reduced to 26.8/cm. The latter is a well known and readily soluble wetting agent, used in one of the standard culture media for coliform detection in water.

The effect of Deceresol-OT on pathogenicity of bacteria for mice was tested with *Shigella dysenteriae* and *Shigella paradysenteriae;* that of Duponol W.A. with *Salmonella typhosa* (strain "Panama 58") and *Shigella ambigua*. Suspensions of the organisms in saline were adjusted to known concentrations according to turbidity standards. Starting from one suspension of each organism, further dilutions were prepared with the same ratio, 2, 4 or 5. In any test, 4 to 9 of these dilutions were used in each of 2 series: the controls, containing the organism in saline and the experimental series, to which a solution of wetting agent was added.

Results. Table I gives the final concentrations of wetting agents used for different organisms and the volumes inoculated intraperitoneally into each animal. Five Swiss albino mice weighing 15 to 18 g were inoculated with each dilution of the control or experimental series. In the latter the mice received 1 mg of wetting agent, except those inoculated with *Shigella ambigua*, which received 1.66 mg of Duponol W.A. Preliminary experiments in which 0.5 ml volumes were inoculated intraperitoneally, showed that mice receiving up to 1 mg of Deceresol-OT only occasionally showed some fur ruffling, whereas amounts up to 2.5 mg of Duponol W.A. were well tolerated. As an additional control of the innocuousness of the wetting agents to the experimental animals, in each test an equal amount of the chemical alone in the same total volume of saline was inocu-

* Trademark, Amer. Cyanamid Co.

TABLE I. Effect of Wetting Agents on Pathogenicity of Enterobacteriaceae Inoculated Intraperitoneally into Mice.

Organism	Chemical and conc.	Vol inoculated, ml	LD_{50}* Exp.	LD_{50}* Control
Sh. dysenteriae	Deceresol-OT 1:500	.5	6.72	8.73
Sh. paradysenteriae	" "	"	6.30	8.63
Salm. typhosa	Duponol W.A. "	"	7.52	8.34
Sh. ambigua	" 1:600	1.0	6.81	8.55

* Logarithms to base 10 of No. of organisms killing 50% of inoculated mice in 72 hr, as estimated by the Reed-Muench method from 4 to 9 groups of 5 mice.

lated intraperitoneally into 5 mice: in no case did these mice show any sign of illness.

The median lethal doses were estimated by the Reed-Muench method, and the results, expressed as logarithms to base 10, are presented in Table I. It is clear that there was considerable reduction in the LD_{50} values for the experimental series of each test as compared with the corresponding control, indicating increases of approximately 100, 200, 7 and 60 times in pathogenicity of, respectively, *Sh. dysenteriae, Sh. paradysenteriae, Salm. typhosa,* and *Sh. ambigua.* The effect of Duponol W.A., though quite definite, seems to be less intense than that of Deceresol-OT. Further experiments are necessary to establish whether the enhancement of pathogenicity by wetting agents is peculiar to enteric bacilli inoculated intraperitoneally into mice as well as to elucidate the mechanism of the observed effect.

Summary. Two wetting agents, Deceresol-OT and Duponol W.A., when added to suspensions of *Sh. dysenteriae, Sh. paradysenteriae, Salm. typhosa,* or *Sh. ambigua,* consistently enhanced pathogenicity of the organisms for mice inoculated intraperitoneally.

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Biochemical and Pharmacological Studies with D- and L-5-Hydroxytryptophan. (23066)

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Following synthesis of 5-hydroxy-D,L-tryptophan (5HTP)(1) it was shown that this amino acid is the precursor of 5-hydroxytryptamine (serotonin)(2) and that this conversion is catalyzed in animal tissues by a specific decarboxylase(3). The amino acid itself has now been found in toad venom(4) in *Chromobacterium violaceum*(5) and in urine of a patient with malignant carcinoid.* It

* C. E. Dalgliesh, personal communication.

has also been reported that D,L-5HTP, when administered to animals, is taken up by tissues, including brain, and there decarboxylated to serotonin(6). Upon its administration 5HTP causes somatic, autonomic and behavioral changes which bear a striking resemblance to those produced by the hallucinogenic agent, lysergic acid diethylamide (LSD). It has been inferred that the pharmacological effects produced by 5HTP result from its conversion to serotonin. From previous studies on conversion of D,L-5HTP to serotonin it was apparent that only one of the isomers was decarboxylated to serotonin(3). It was therefore concluded that the observed pharmacologic effects following administration of D,L-5HTP were due to that antipode which could be decarboxylated to serotonin, presumably the L form. It has now been possible to obtain evidence that only the L form is converted to serotonin and that this conversion accounts for previously reported pharmacologic effects of D,L-5HTP(6).

Methods and materials. 5-Hydroxy-D,L-tryptophan and 5-benzyloxy-D,L-tryptophan were kindly supplied by Dr. Kenneth E. Hamlin of Abbott Laboratories and Dr. Gustav J. Martin of National Drug Co. The L-amino acid oxidase used was a crude dried rattlesnake venom extract obtained from Ross Allen Farms (*Crotalus adamantus*). The carboxypeptidase used was a purified preparation kindly supplied by Dr. John E. Folk. The hog kidney acylase was a commercial preparation kindly supplied by Dr. Leon Levintow. Both preparations were shown to be active when tested on carbobenzyloxyglycylphenylalanine. *Serotonin* in tissues was assayed by a method previously described(7). 5HTP decarboxylase activity was determined by the method of Clark *et al.*(3). L-amino acid oxidase activity was determined by measuring the generated 5-hydroxyindoleacetic acid† (5HIAA) as previously described(8).

Results. Enzymatic resolution of D,L-5HTP was attempted. However, solutions of N-acetyl-5-benzyloxy-D,L-tryptophan and of N - chloroacetyl-5-benzyloxy-D,L-tryptophan, when incubated with carboxypeptidase at 37° for 16 hours did not lead to ninhydrin positive material(9). The same negative result was observed when hog kidney acylase was employed (10). Attempts to prepare salts of optically active acids with esters of 5-benzyloxy-D,L-tryptophan were discontinued when it was found that debenzylation occurred during Fischer esterification. Resolution of N-acetyl-5-benzyloxy-D,L-tryptophan was successfully accomplished by recrystallization of the brucine salt from 95% ethanol, as indicated by attainment of constant optical rotation ($(\alpha)^{D}_{20}$-6.1, c, 1.0 ethanol). However, deacetylation without racemization or destruction was impossible.

It was found possible to demonstrate resolution of the quinine salt of N-carbobenzyloxy-5-benzyloxy-D,L-tryptophan (m.p.-132°).‡ A solution of this salt was prepared by dissolving 4.4 g of N-carbobenzyloxy-5-benzyloxy-D,L-tryptophan and 3.2 g of quinine in 100 ml of 95% ethanol. On cooling there was deposited 2.1 g of crystals which were recrystallized twice from the same solvent to yield 0.8 g of crystalline salt. The salt was decomposed by the addition of excess 2 N HCl and extraction of the indole component with 100 ml of ethyl acetate. The organic solvent when evaporated left 0.42 g of one antipode of N-carbobenzyloxy-5-benzyloxytryptophan, yielding after hydrogenolysis 120 mg of the crystalline amino acid (Fraction A containing antipode D). In order to obtain the other antipode the mother liquor (100 ml of 95% ethanol) from the first crystallization of the quinine salt, was concentrated to 70 ml, left standing overnight at 5°C and filtered from additional D-salt. The filtrate, after removal of the quinine and hydrogenolysis of the

† Since these preparations are devoid of catalase the keto acid is nonenzymatically decarboxylated by the peroxide. 5-Hydroxyindole-pyruvic acid does not differ from 5-HIAA with regard to color reactions and extraction pattern.

‡ While this investigation was in progress Dr. Marvin Armstrong kindly sent us a manuscript (*J. Org. Chem.* in press) in which he described the successful resolution and the isolation of the two antipodes of 5HTP in chemically and optically pure form, using the quinine salt of N-carbobenzyloxy-5-benzyloxy-D,L-tryptophan.

TABLE I. Enzymatic Reactivity of D,L and D and L-5-Hydroxytryptophans.

Enzyme	Sample	% conversion	
L-amino acid oxidase*	DL-5HTP	49	5HIAA
	Fraction B (L)	80	
	" A (D)	0	
5HTP decarboxylase†	DL-5HTP	50	Serotonin
	Fraction B (L)	82	
	" A (D)	3	

* 4 mg of snake venom were incubated with 100 μg of either D or L 5HTP or with 200 μg of D,L-5HTP, in 3 ml at pH 7.4 and 37°C for 1 hr. Deamination was complete within this period.

† 2 ml of a 100,000 × g guinea pig supernate were incubated with 300 μg of each antipode in 3 ml at pH 8.1 and 37°C for 1 hr. Decarboxylation was complete within this period.

benzyl groups, yielded 100 mg of crystalline amino acid (Fraction B containing mainly antipode L). Due to the paucity of the isolated material further purification by recrystallization was not attempted. By comparing molecular extinctions of the α-nitroso-β-naphthol chromophores(11) with that of a standard preparation of 5-hydroxy-D,L-tryptophan, Fractions A and B indicated a purity of 80-85% with regard to 5HTP; this was taken into account in the following experiments.

Fractions A and B when subjected to analysis by paper chromatography indicated only one indole component identical with authentic D,L-5HTP. Evidence concerning the optical identity of the two resolved antipodes and of the optical purity of each is presented in the section on enzymatic studies.

Enzymatic studies. L-amino acid oxidase: Fractions A and B and D,L-5HTP were incubated with L-amino acid oxidase permitting the reaction to go to completion. As shown in Table I the racemic mixture yielded 49% of the theoretical amount of 5HIAA. Fraction B yielded 80% of the theoretical amount of 5HIAA whereas no 5HIAA was produced from Fraction A. Thus Fraction B was taken to be the L form. Apparently it still contained some of the D-antipode. However, Fraction A was apparently the pure D-antipode. 5HTP was found to be a much poorer substrate of hog kidney D-amino acid oxidase than alanine. *5HTP decarboxylase*: When the various forms of 5HTP were treated with crude preparations of guinea pig kidney decarboxylase the racemic mixture yielded 50% of the theoretical amount of serotonin (Table I). Fraction B was also decarboxylated and reacted to a similar extent as it had with L-amino acid oxidase. Little serotonin was formed from Fraction A.

These enzymatic findings identify the configurations of the two antipodes and substantiate the optical specificity of 5HTP decarboxylase. *Pharmacological studies.* The pharmacological effects produced by 5HTP, in previous studies(6) were explained by the production of serotonin *in vivo*. However, one could not rule out the possibility that some of the observed actions might be due to 5-hydroxytryptophan itself.

With the resolution of 5HTP it became possible to compare the pharmacological activities of D and L forms only one of which can be enzymatically converted to serotonin. Each of the isomeric forms of 5HTP and the racemic mixture were administered to mice. After 15-20 minutes those animals given the L form (Fraction B), or twice the amount of D,L-5HTP, exhibited marked tremors, piloerection, apnea, and hind limb abduction. After 35 minutes most of these animals were moribund. In contrast, those animals which had been given the D form (Fraction A) did not differ from the control animals. At the end of 45 minutes all the animals were sacrificed. The brains from all 4 animals in each group were pooled, homogenized and assayed for serotonin. The results are shown in Table II. Consistent with their observed central effects L and D,L-5HTP produced a large increase in brain serotonin. No increase in serotonin appeared in those animals

TABLE II. Serotonin Levels in Brain following Administration of the Various Forms of 5HTP.

Exp.	Brain serotonin, mg/g
Control	.8
D,L-5HTP	5.0
L- "	3.8
D- "	.8

Mice were pretreated with 10 mg of iproniazid phosphate (i.p.) 40 min. before administration of 2.5 mg of 5HTP (i.p.). They were sacrificed 45 min. after receiving 5HTP.

treated with the D form.

Discussion. The resolution of 5HTP into the D and L forms has permitted establishment of the optical specificity of one of the enzymes involved in the biogenesis of serotonin, 5HTP decarboxylase. It has also made it possible to further substantiate the claim that the central effects of 5HTP are due to its *in vivo* conversion to serotonin. It may also be of some interest that the anti-insulinase activity previously reported for D,L-5HTP has likewise been shown to be restricted to the L antipode.§

Summary. The D and L forms of 5-hydroxytryptophan were prepared. Only the L form was found to be acted on by 5HTP decarboxylase, to increase brain serotonin levels *in vivo*, and to be pharmacologically active.

§ Dr. Martha Vaughan, personal communication.

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Relation of Adrenal Weight to Social Rank of Mice. (23067)

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Recent work(2) has shown that mice in groups have significantly heavier adrenal glands than mice kept in isolation. Other work has shown that mice in a group will arrange themselves in an ordered social rank such that each mouse is dominant over those below it in rank(4,5). It seemed logical to investigate whether or not there is a relationship between social rank and adrenal weight. This report describes experiments designed to determine if there is such a relationship, after first performing pilot experiments to determine if the procedures used would in themselves produce changes in adrenal weight.

Methods. The mice used were "wild-strain" house mice (*Mus musculus*). These were descendants of wild mice captured in buildings and had been maintained in the laboratory for more than 10 generations. This strain is superior to albino mice for the present purpose, because past work(2) has shown that their adrenals and reproductive organs are more responsive to stressing stimuli. Male mice were weaned at 20 days and placed individually in jars, where they were kept until becoming sexually mature, usually at an age of 2 months and weight of 20-25 g. The jars were kept on shelves with partitions between the jars. Thus the mice were isolated visually, but not auditorily or olfactorily. The experimental design called for placing mice in groups for 4 hours each day, observing the social rank of each individual and then returning each mouse to its own jar for the remaining 20 hours of the day, and repeating this procedure daily. It was therefore important to determine by a pilot experiment whether daily handling would itself produce increase in adrenal weight and

TABLE I. Pilot Experiment on Procedures.

Treatment for 10 days	n	Body wt, g*	Adrenal wt, mg*
A. Isolated but not handled	16	25.9 1.1	4.49 .77
B. Isolated but change jar daily	7	25.7 .7	4.49 .61
C. Grouped for 4 hr a day	7	25.7 2.5	5.48 .54
D. Grouped continuously	7	26.0 1.0	5.38 .79

* Mean and S.D. given.

whether being grouped only 4 hours a day would be as effective in producing adrenal enlargement as leaving the mice continuously in groups. The pilot experiment (Dec. 1954) consisted of comparing the adrenal weights of 4 groups of mice. One group (A) consisted of 16 male mice which were left in isolation without handling. A second group (B) consisted of 7 mice which for 10 days were kept in individual jars, but transferred from one jar to another for 4 hours and then returned to their original jar. The third group (C) had 7 mice which for 10 days were placed together each day in a 20 gal. metal container for 4 hours and then returned to their individual jars for the remaining 20 hours. The final group (D) were left grouped continuously in a 20 gal. metal container for 10 days. After the 10 days of experimental handling all mice were sacrificed and their adrenal weights obtained and compared. The results (Table I) showed that handling isolated mice twice a day did not alter adrenal weight and that the adrenal weights of the grouped mice (C and D) were significantly greater than of isolated mice (groups A and B). No significant difference in adrenal weight was noted irrespective of whether the mice were grouped for 24 or only 4 hours a day. It was considered desirable to give the mice 20 hours in their own private jar to recuperate and feed. The results of the pilot studies indicated that this procedure could be followed. On the basis of the above results and accumulated experience, the following routine was fixed for subsequent experiments. For practical reasons the number of mice per group was reduced from 7 to 6. Six male mice were randomly chosen and kept in their visually isolated individual jars. Selection of mice for each experimental group was made by choosing groups of mice with body weights as homogeneous as possible. The weights sometimes varied as much as 6 g within a group, but this difference was not considered important in mature mice since all adrenal weights were to be expressed in terms of mg/100 g body weight. The 6 mice in each group were placed in a 20 gal. metal can for 4 hours daily (except Sunday) for 10 days. The basal area of the container was 6 times that of the individual jars. The floor of the can was covered with sawdust. At first food and water were supplied, but these were eliminated because the mice rarely fed or drank when grouped. Usually 2 groups were run simultaneously and all experiments were done from December to March in 1954, 1955, and 1956. Social rank was determined by observation of fighting. Each mouse could be identified by the position of a shaved patch of skin, so the identification of the victor and loser in contests was possible. Usually the top-ranking mouse was obvious, even on the first day. Rank determination was further confirmed on the last day of the experiment by a round-robin of contests between pairs of mice. Usually rank was clearly determined by the latter procedure, although ties did occur occasionally. There was no indication that the rank as displayed in a paired comparison differed from the rank when the same 2 mice were part of the larger group. In every group the most dominant mouse (rank I) was obvious. Usually the second and sixth ranks were also easily determined, but it was frequently difficult to separate the third ranking mouse from the fourth, or the fourth from the fifth. This difficulty was due to a lack of fighting rather than reversals or ties. Thus assigned rank may be erroneous for mice in the middle of the hierarchy and in some ways it would be better to treat mice in ranks III, IV and V as a group rather than as 3 separate ranks. It should be kept in mind that these ranks are not necessarily equally spaced within a group or between groups. Occasionally rank changed during the 10 days and usually was a reversal between the 2 top mice in the first few days. In

TABLE II. Results of Social Rank and Adrenal Weight Determinations for 18 Groups of 6 Mice Each. (Body wt in g. Adrenal wt in mg/100 g of initial body wt. Social rank (Roman numerals) is non-parametric.)

Rank	I		II		III		IV		V		VI	
Group	Body wt	Adrenal wt	Body wt	Adrenal wt	Body wt	Adrenal wt	Body wt	Adrenal wt	Body wt	Adrenal wt	Body wt	Adrenal wt
B	30.	15.7	28.	19.6	30.	17.5	30.	19.2	30.	20.7	28.	17.3
C	30.	17.9	30.	16.7	28.	17.3	30.	23.7	28.	18.3	30.	17.7
D	28.	13.	26.	17.1	28.	18.1	26.	19.2	28.	22.9	28.	17.8
E	28.	18.9	28.	19.1	28.	8.6	26.	13.3	26.	20.7	28.	17.1
F	32.	18.3	34.	18.4	34.	21.1	32.	12.3	32.	15.9	26.	14.3
G	30.	15.4	30.	20.6	24.	22.	24.	19.9	24.	21.6	30.	19.7
H	20.2	23.2	19.	26.5	23.5	26.9	20.	26.9	21.5	17.6	23.7	20.4
I	25.	19.8	21.5	21.1	22.8	24.3	21.9	24.3	23.	23.1	22.6	26.7
J	23.9	20.2	25.5	23.6	22.5	24.2	26.1	22.3	26.1	23.7	22.	27.5
K	24.6	23.9	23.8	23.2	24.3	21.5	23.1	28.4	24.5	22.4	23.5	27.1
L	24.8	21.7	24.1	19.6	24.9	20.5	21.9	31.1	23.3	33.2	21.3	21.9
N	24.9	22.8	22.6	15.9	24.5	19.	23.4	22.8	22.6	24.2	20.3	21.3
O	24.8	23.3	24.5	25.5	25.2	21.8	22.8	21.4	25.2	21.	23.	29.9
P	25.4	21.8	24.8	23.	23.8	22.1	25.3	19.3	23.	27.1	23.8	24.
Q	25.2	18.5	22.2	22.9	24.	20.4	23.1	21.9	25.1	19.8	25.6	21.6
R	22.5	20.9	24.	24.7	23.6	17.	23.3	23.	22.1	17.4	25.1	26.7
S	24.5	17.8	26.1	19.	24.6	19.2	24.2	17.9	23.7	17.9	24.1	22.
T	24.3	20.	22.8	18.9	25.8	21.5	24.8	19.2	24.4	19.3	24.8	22.7
Mean	26.	19.6	25.4	20.9	25.6	20.2	24.9	21.5	25.1	21.5	25.	22.

other cases a mouse became ill and declined in rank. Rank on the tenth day was always used for the results. The mice were killed by a blow, weighed, and the adrenals removed and fixed in formalin. They were later weighed on a Roller-Smith torsion balance. Since rank was assigned without any measure of the degree of difference between ranks, a non-parametric method of statistical analysis of the relationship between rank and adrenal weight had to be used. The "ordering test" described by Quenouille(3) was used.

Results. The details of each experiment are given in Table II. Body weight is the initial weight at the beginning of the experiment. Adrenal weights are given as mg/100 g of initial body weight. The initial body weight is preferred rather than the final weight, as in most cases rank was decided on the first or second day and in addition does not reflect losses in weight during the 10 days. The results are shown graphically in Fig. 1. It is apparent that all but the top-ranking mouse (I) lost weight during the 10-day period of grouping. It is equally apparent that adjustment of adrenal weight to final body weight could suggest a greater increase in adrenal weight than was actually the case.

The order test(3) gives a significance of $P<0.01$ for the relationship between social rank and adrenal weight.

On the basis of this analysis and graphical presentation of the data, it is concluded that

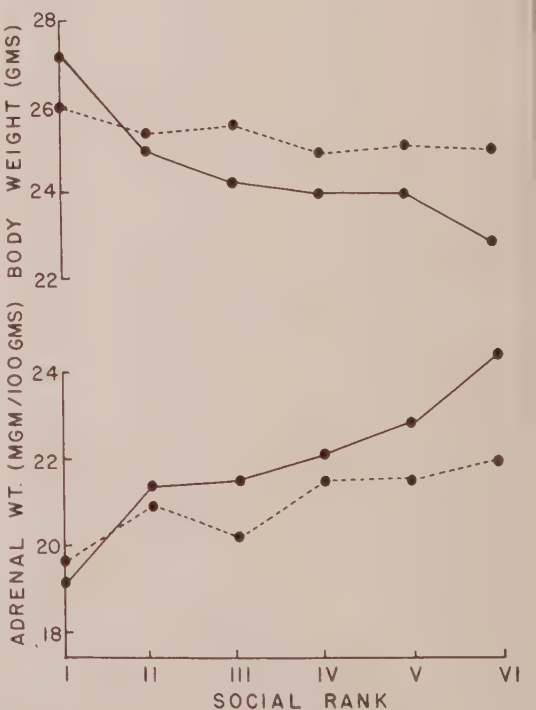

FIG. 1. Relation of body weight and rank (upper lines) and adrenal size and rank (lower lines). Dotted line refers to weight at start and solid line refers to weight at end of 10 days.

low-ranking mice have larger adrenals at the end of 10 days under these experimental conditions than do high-ranking mice. The biological significance appears to be that low-ranking mice are subjected to more physical and psychological stressing stimuli. Barnett (1) noted that the function and size of the adrenal cortex was greater in subordinate rats than in dominant rats.

Summary. Mature male "wild-strain mice," isolated from weaning, were placed in groups of 6 for 4 hours a day for 10 days. The social or dominance rank of each mouse was determined. Adrenal and body weights were obtained for each mouse. A significant relationship was found between adrenal weight and social rank. It is concluded that high-ranking mice have smaller adrenals than do low-ranking mice.

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Role of Estrogen in Sex Difference of the Electrocardiogram of the Chicken.* (23068)

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Data from this laboratory show a sex difference in the blood pressure and heart rate of chickens(1) but not in the pigeon and duck(2). It was also shown that estrogen played a role in the blood pressure difference (3). Earlier studies on the electrocardiogram of chickens by Kisch(4) and Sturkie(5) were not concerned with a possible sex difference as their experiments dealt with only one sex, or sex was not indicated.

This report shows that the amplitude of the male chicken ECG is considerably greater than that of the female and that administration of estrogen to the male decreases amplitude significantly.

Procedure. The data pertaining to the normal sex difference in amplitude of ECG are based on records of yearling males and females taken in March and June of 1955. The estrogen experiments were conducted about one year later on yearling males fed a standard commercial diet. ECG's were run on males before, during and 2 months after discontinuance of estrogen treatments. Control ECG's were taken, and one week later the males received one 15-mg pellet of diethylstilbestrol as an implant. Two weeks later another 15-mg pellet was implanted. The second ECG was taken 29 days after the first pellet was implanted. The birds then received a third pellet, and the third ECG was taken 14 days later or 43 days after estrogen was first administered. The last ECG was taken 57 days after the last administration of estrogen. Based upon comb size, the birds appeared to have recovered from estrogen.

ECG's were recorded on unanesthetized birds, using a Sanborn 150-1600-amplifier and a 151-100A recorder, at a chart speed of 50 mm per second. Needle electrodes were inserted in the muscles at the base of right and left wings and the muscle of the left thigh, to form the standard limb leads(5). Amplitudes of 5 or more R and S waves per bird were measured on all ECG's of leads II and III and the averages recorded. P waves of a few selected records were measured, but it is difficult to measure amplitudes of P and T waves because they are often fused in the chicken ECG(6). To measure amplitude accurately at the usual standardization of 1

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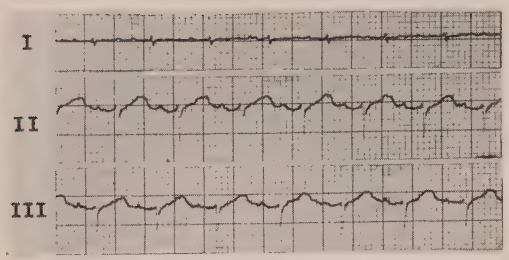

FIG. 1. Leads I, II and III of ECG of normal female chicken. Standardization, 1 cm equal 1 mv, chart speed 50 mm per sec.

cm = 1 mv, the records were magnified so that each unit of measurement equaled ¼ mm or 1/40 mv. RS axes were calculated from leads II and III.

Results. ECG's of normal males and females. The chicken ECG exhibits P, S and T waves and usually a small, abortive R wave but no Q. The R is more prominent in the male but less so than S (Fig. 1 and 2). The amplitude of all waves of lead I is low, and the T wave is usually isoelectric. P and T waves are often fused in the female record, even when recorded on instruments of high frequency (500 cycles per second) and rapid chart speeds (50-75 mm per second). Rarely is this true of the male ECG.

The amplitude of all waves of the male ECG is considerably greater than that of the female. The magnitudes of differences for R, S and RS are shown in Table I, where the amplitude for the male is over twice that of the female. The differences are highly significant statistically.

ECG's of estrogen-treated males. Estrogen

TABLE I. Mean Amplitude in Millimeters of R, S, and RS in ECG, Leads II and III, of Adult Chickens. Standardization, 1 cm = 1 mv.

	II			III		
	R	S	RS	R	S	RS
			25 males			
Mean	2.65	7.95	5.30	1.82	7.72	5.90
S. D.	1.44	2.22	2.93	1.22	2.51	2.43
S. E.	.30	.45	.60	.25	.51	.49
			31 females			
Mean	.83	3.30	2.47	.30	2.80	2.50
S. D.	.81	.85	1.21	.47	.87	.99
S. E.	.15	.16	.22	.09	.16	.18
Mean diff.	1.82	4.65	2.83	1.52	4.92	3.38
t*	5.50	6.00	9.70	9.10	4.40	6.30

* All P values <.01.

TABLE II. Mean Amplitude in Millimeters of RS Complex of ECG of Males (11 to 13) before and after Estrogen Administration. Standardization, 1 cm = 1 mv.

Lead	Before estrogen	During estrogen 29 days	During estrogen 43 days	After estrogen
II	9.	5.70	5.99	7.43
III	10.	6.47	6.43	7.86

administration resulted in a considerable decrease in the amplitude of P, R, and S waves and some decrease in T (Fig. 2). The RS complex is characterized usually by a small R and a prominent S wave. Thus, the changes in R and S resulting from estrogen represent changes mainly in S. The mean amplitudes of RS before, during, and after recovery from estrogen are shown in Table II, and percentage change in Table III. The decrease in RS between treatments 1 and 2, and 1 and 3 (before and during estrogen) in leads II and III range from 33.5 to 36.8%. ECG's run on the untreated birds during these periods exhibited no change. Fifty-seven days after estrogen was administered, or approximately 1 month after all estrogen had been absorbed, (treatment 4), the amplitudes of RS increased from 20 to 30%, but were still less than before estrogen. Although

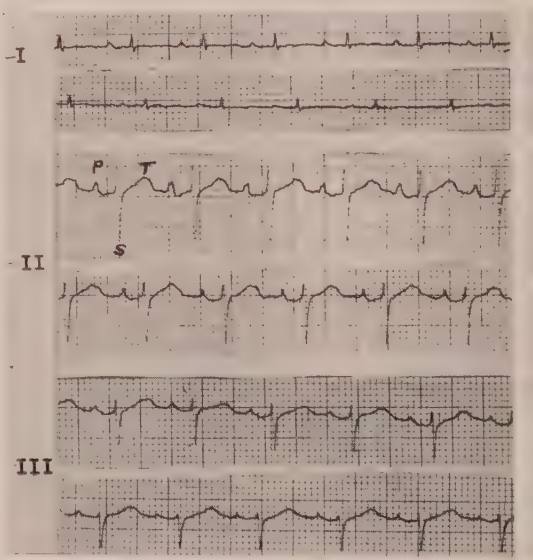

FIG. 2. Leads I, II and III of male chicken before (upper) and after (lower) estrogen administration. Standardization, 1 cm equal 1 mv, chart speed 50 mm per sec.

TABLE III. Percent Difference in Mean RS Complexes of Males before Estrogen (1), during Estrogen (2 and 3) and after Estrogen Administration (4). (Differences paired.)

Treatments, % change from-to	Lead II			Lead III		
	Diff.	Error	t*	Diff.	Error	t*
1 & 2	−36.80	2.04	6.5	−36.05	1.97	7.4
1 & 3	−33.53	2.07	5.4	−36.34	2.17	6.3
1 & 4	−17.64	2.35	3.5	−22.13	2.23	3.7
2 & 4	+30.00	1.83	3.2	+21.68	1.92	3.4
3 & 4	+19.50	1.78	2.2†	+22.25	1.88	2.8

* All t values highly significant except (†) which is borderline.

the ECG's showed a significant recovery from the effects of estrogen it was not complete, because the values were still significantly less than pretreatment values (difference between 1 and 4).

RS axes. The mean electrical axes for the 4 treatments were: 1) 85.8, 2) 82.46, 3) 87.16 and 4) 85.2 degrees. None of the differences is significant.

P wave. The amplitude of the P waves, based on a few selected records, showed a decrease of 21.0% resulting from the estrogen treatment.

Heart Rate was not influenced by estrogen and this confirms our previous findings(3).

Discussion. These data demonstrate that the amplitude of R, S and RS in the normal female chicken ECG is approximately 55% less than that of the male. The voltage of the ECG of the estrogenized male is 30 to 35% less than in normal males. These results might suggest that estrogen plays a role in the normal sex difference of chicken ECG. Laying hens secreting estrogen and estrogenized males exhibit a phenomenal increase in blood lipids and a tendency to obesity(6), which may be conducive to the lower voltage of chicken ECG, as it has been reported to be for the human ECG(7), because adipose tissue tends to shunt or resist cardiac electropotentials(7,8). Whether or not obesity influences voltage of the chicken ECG, and if so, to what extent such changes are influenced by input impedance of amplifier used remains to be determined.

Estrogen at the level administered in this experiment to males depresses size of comb and testes and suppresses the output of pituitary gonadotrophins and androgen. Thus, the decreased amplitude of the estrogenized male ECG could result from lack of androgen rather than the effects of estrogen, and likewise the normal sex difference in ECG may be attributable to androgen rather than estrogen. Further studies on sexually immature males and females and castrates should reveal which gonadal hormones if any, are operative and to what extent.

Summary. The data demonstrate that amplitudes of R, S and RS of the ECG of normal hens are approximately half those of normal males. The effects of estrogen on the ECG of adult males were studied. The voltage of these waves of estrogenized males is decreased about 30 to 35%.

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Active Constriction and Dilatation in Pulmonary Circulation in Response to Acetylcholine.* (23069)

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Many believe that the pulmonary circulation is passive, in that changes in vessel calibre are due only to changes in blood flow or alterations in intrapulmonary blood volume. The observed effects of drugs on pulmonary arterial pressure have been attributed to back-pressure from the left auricle(1), redistribution of blood volume(2) or altered bronchomotor tone(3). However, recent reports (4,5,6) of experiments that excluded extrapulmonary factors indicate that vessels of the lesser circulation are capable of active vasoconstriction in response to appropriate pharmacologic stimuli. Older experiments showed that acetylcholine produces vasomotor responses in isolated pulmonary vessel segments (7) and perfused resected lungs(8).

The present report is concerned with the use of this drug in demonstrating ability of lesser circulation to actively dilate, and in investigating relationships between pulmonary vasomotor and bronchomotor activity.

Methods. A diaphragm type of pump was substituted for the left ventricle in dogs. The apparatus and technic for establishment of this extracorporeal circulation have been described in detail(9). Blood was drained totally from left auricle via a 3/8 I. D. Tygon tube to a reservoir. From the reservoir it was pumped to a T-tube in the descending thoracic aorta. The left ventricle thus was by-passed. The right ventricle continued to function effectively, keeping pace with the mechanical pump. Stroke volume and rate of "left ventricular" pump were kept constant at levels between 1.8 and 2.4 l/minute. Pump output does not vary with alterations in peripheral vascular resistance(9). The modification by which effects of drugs in the pulmonary circulation were studied, has also been described(6). As the drug under study was injected into the main pulmonary artery, the tube draining the left auricle was diverted to a second reservoir. The systemic circulation continued to be maintained with blood from main pump reservoir. When main pump reservoir was near depletion, the left auricular tube was returned to its original position. Then, after an interval, the blood and drug in the temporary container were returned to the system. During period in which the left auricular tube was diverted from main pump reservoir, the drug traversed only the lungs and was excluded from the systemic circulation and ventricles. All studies were controlled with saline injections of comparable volumes. Acetylcholine was injected in volumes never greater than 1 ml. In several instances, instead of direct pulmonary arterial injection, the drug was introduced through a polyethylene catheter, the tip of which was placed in the right ventricle. This was to obviate any "streamlining" effect with unequal distribution to various portions of the lungs. No qualitative or quantitative differences could be noted between intraventricular and direct pulmonary arterial injection. Mongrel dogs weighing 12 to 18.5 kg were anesthetized with sodium pentobarbital 25 mg/kg. All studies were made in open-chest animals. The trachea was intubated and respirations were maintained with 100% oxygen delivered via variable phase pulmotor valve. In nebulization experiments, acetylcholine was delivered into airway in 1% concentrations with Vaponephrin® nebulizer under pressure, through 4 or 5 respirations. Experiments were controlled with nebulizations of normal saline. Cervical vagotomies performed in 3 experiments were bilateral. Methods for pressure and flow recording have been previously described(9). In the present experi-

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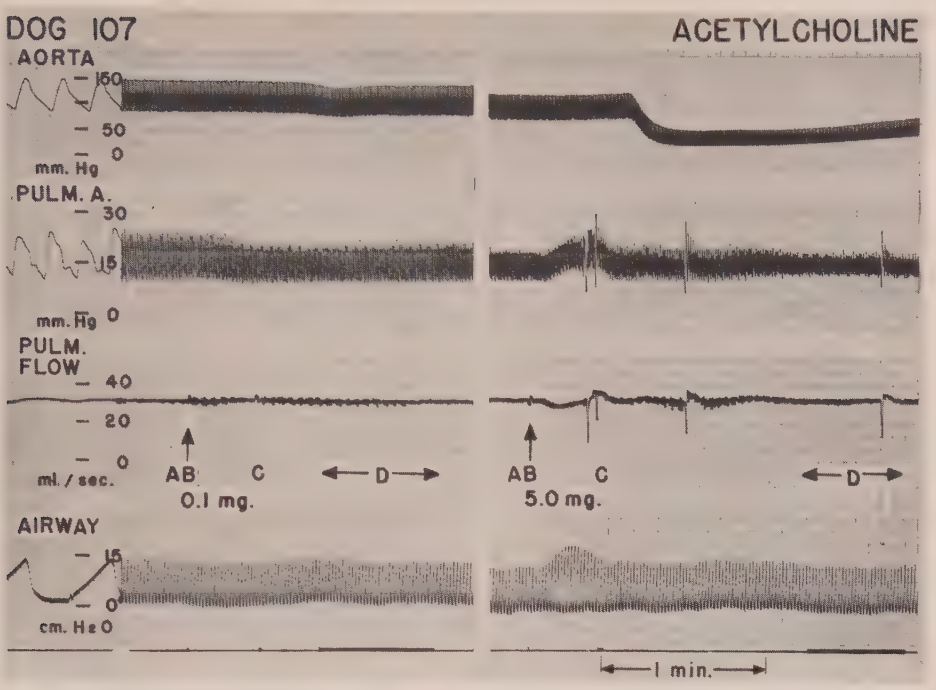

FIG. 1. Original records of injection of 8 μg/kg (left) and 400 μg/kg (right) acetylcholine into main pulmonary artery. At (A), left auricular tube was diverted to temporary reservoir. At (B), injection was made. At (C), left auricular tube was returned to its original position. Between (B) and (C), the drug traversed only the lungs. At (D), blood in the temporary reservoir, which contained the drug that had traversed the lungs, was returned to the experimental system. The smaller dose produced a fall in pulmonary arterial pressure and a slight increase in flow with no change in airway pressure. The larger dose caused a rise in pulmonary arterial pressure and a fall in flow, with an elevation of airway pressure. The fall in aortic pressure following (C) on right occurred before the drug in temporary reservoir was returned to circulation and was probably due to small amounts of acetylcholine still washing out of the lungs.

ments. left auricular blood flow was monitored with a Shipley-Wilson rotameter.

Results. Acute effects of acetylcholine injected into pulmonary artery. Preliminary screening of many drugs in this experimental preparation revealed that acetylcholine was a particularly potent agent for increasing pulmonary vascular resistance. However, since these observations were made only on vascular pressures and flow, and since Rodbard(3) has suggested that the acetylcholine response is due to altered bronchomotor tone, these experiments were repeated with continuous monitoring of intratracheal (airway) pressure. The following data are taken from observation of 31 injections of acetylcholine in 10 experimental preparations.

In doses of 0.1 to 5 mg (9 to 400 μg/kg) acetylcholine produced increased pulmonary vascular resistance (6 to 150% of control value) in 13 experiments. An example of this response is shown in the record on the right side of Fig. 1. In each instance, the increased pulmonary vascular resistance was accompanied by acute rise in airway pressure of variable magnitude.

On the left side of Fig. 1, the record is representative of the response noted in 18 experiments in which 0.01 to 0.15 mg (0.5 to 10 μg/kg) acetylcholine were injected into the pulmonary artery. In these instances, little or no rise in airway pressure was detectable, and pulmonary vascular resistance fell 6 to 29% of control value.

In neither of the above groups was it possible to establish a dose-response relationship. However, in individual experimental preparations, it was possible to clearly draw the line at which the pulmonary depressor response changed to a pressor response. The minimal

dose at which a pressor response was noted ranged between 9 and 70 μg/kg acetylcholine injected in the pulmonary artery. A point at which no resistance alteration resulted because of balancing of opposing effects was never detected. Varying experimental conditions and gradual development of tachyphylaxis, especially to smaller doses, may have contributed to the inability to quantitate these effects any further.

Response to acetylcholine following bilateral cervical vagotomy. Respiratory and vascular reflexes mediated by the vagi have been demonstrated as arising from pulmonary blood vessels in response to a variety of chemical(10) and mechanical(11) stimuli. These have been demonstrated with the present experimental preparation(12). Following bilateral cervical vagotomy in 3 dogs, the pulmonary vasopressor and bronchomotor responses to acetylcholine (60 to 200 μg/kg) injected into the pulmonary artery were unaltered. This indicates that the observed vascular and bronchial responses probably were directly elicited by the drug rather than by reflex stimulation.

Reexamination of effects of pressor amines with respect to bronchomotor activity. Increased pulmonary vascular resistance in response to epinephrine and norepinephrine was demonstrated by the same technic(6). However, airway pressure was not recorded. In 3 dogs, norepinephrine was retested in doses of 6 to 12 μg/kg injected into the pulmonary artery. In no instance was there an increase in intratracheal pressure. In one case, there was a fall in airway pressure simultaneous with increased pulmonary vascular resistance.

Effects of nebulized acetylcholine on pulmonary vascular resistance. Introduction of 1% acetylcholine into the airway via the nebulizer produced an average rise in maximum inspiratory airway pressure of 39% (range 15 to 80%) in 6 experiments in 5 preparations. The maximal rise occurred between 25 and 110 seconds following nebulization. There was no rise in pulmonary arterial pressure or alteration in pulmonary venous flow in any instance at time of maximal airway pressure increase.

Discussion. The means by which acetylcholine injected in the pulmonary artery affects the bronchi remains obscure. Marchand, Gilroy and Wilson(13) demonstrated anastomoses between the pulmonary and bronchial circulations in humans, and these have been discussed in relation to the dog pulmonary circulation by Daly *et al.*(14). It is possible that acetylcholine enters the bronchial capillary or venous circulation via such pulmonary-bronchial anastomoses, so that bronchomotor effects could occur even when the pulmonary circulation is isolated from the systemic circulation. On the other hand, it is possible that respiratory units provided with sufficient smooth muscle to produce significant elevations of intratracheal pressure are supplied by the pulmonary artery.

The relationships between bronchomotor and pulmonary vasomotor activity remain to be further clarified. It remains to be determined whether intratracheal pressure accurately reflects pressure throughout all portions of the bronchial tree.

The qualitatively different pulmonary vessel response to large and small doses of acetylcholine was demonstrated by Gaddum and Holtz in perfused lungs(8). In the present studies in which normal pressures, flows, and ventilation were maintained, the older findings were confirmed. The recent investigation of Harris and his colleagues(15) suggests that acetylcholine produces vasodilatation in the human pulmonary circulation and that this effect is augmented by preexisting increased tone in the pulmonary vessels. None of the investigations cited suggest which are the most active segments in the lesser circulation.

Summary and conclusions. The effects of acetylcholine on pulmonary vascular bed of intact dogs were studied in an experiment in which the drug could be excluded from the systemic circulation. While larger doses (usually greater than 0.1 mg) injected into the pulmonary artery were followed by increased pulmonary vascular resistance, smaller doses lowered pulmonary vascular resistance. Exclusion of extrapulmonary factors indicated active pulmonary vasoconstric-

tion and vasodilatation. The larger doses also caused bronchomotor activity. However, the lack of intratracheal pressure changes with pulmonary vasodilating doses of acetylcholine or vasoconstricting doses of norepinephrine, and the absence of pulmonary hypertension with nebulization of acetylcholine indicated that pulmonary vascular resistance alterations were not dependent upon changes in bronchomotor tone.

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Molybdenum in Poult Nutrition.* (23070)

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Molybdenum has been reported to be associated with xanthine oxidase in the rat(1, 2). The maximum level of molybdenum required for optimum xanthine oxidase activity was estimated by De Renzo(3) to be 0.2-0.3 μg/day; however, rate of growth of weanling rats was unaffected by molybdenum supplementation. It was reported by Reid *et al.* (4) that addition of molybdenum to purified diets for chicks or poults resulted in stimulation of growth rate similar to that obtained with distillers dried solubles ash. Remy and Westerfeld(5) were unable to demonstrate an effect of liver residue on xanthine dehydrogenase in the chick as had been obtained in the rat. Higgins *et al.* obtained molybdenum deficiency through addition of 4.5-9.4 mg% tungsten in the ration of chicks. Feeding of molybdenum restored xanthine dehydro-

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genase to normal and corrected other deficiency symptoms. The ash of distillers dried solubles, dried whey and fish solubles has been shown to stimulate growth of both chicks and poults fed purified type diets(6,7) and chicks under practical conditions(8). The present study was initiated to determine effect of feeding distillers dried solubles ash and molybdenum on xanthine dehydrogenase activity in the turkey poult.

Methods. Broad Breasted Bronze turkey poults obtained from dams fed a breeder ration containing alfalfa meal, fish solubles and dried whey were used. The poults were divided into groups of 10 birds at 1 day of age and 2 replicates were fed on each dietary treatment. The basal diet was the same as reported by Reid *et al.*(4) and consisted of soybean protein (Drackett C-1) and starch supplemented to meet requirements of the turkey poult. Poults were maintained in batteries throughout the 4 week experimental period. Feed and water were supplied *ad libitum.* Ten birds from each treatment (5 birds/replicate) were sacrificed for xanthine dehydrogenase determinations on liver and intestine according to the procedure of Remy *et al.*(9). In addition, the left tibia was removed for bone ash determinations. Molybdenum determinations on livers, bones and diets were carried out according to the procedure of Bertrand(10) and Marmoy(11).

Results. Addition of 6% distillers dried solubles to the basal diet produced a 14.9% ($P < 0.05$) increase in growth rate of turkeys at 4 weeks of age (Table I). A similar response (14.4%) was obtained with 0.0254 ppm added molybdenum. This level of molybdenum was found to correspond to one-fourth of that supplied by distillers dried solubles. Distillers dried solubles used in this study was assayed and contained 1.8 ppm molybdenum. The ash of distillers dried solubles was approximately twice as active as the untreated product in stimulating growth of poults (Table I). These data indicate that a portion of molybdenum or other minerals present in distillers dried solubles was unavailable to poults for growth. A mixture of distillers dried solubles, dried whey, fish solubles, an antibiotic fermen-

TABLE I. Effect of Molybdenum, Distillers Dried Solubles and Ash of Distillers Dried Solubles on Growth, Bone Ash and Xanthine Dehydrogenase in Turkey Poults at 4 Weeks of Age.

Supplements to basal diet	Molybdenum content of diets, ppm	Avg wt at 4 wk, g	Response over basal, %	Bone ash, %	Molybdenum content of bone ash, ppm	Xanthine dehydrogenase activity, mm³ O_2/20 min./283 mg tissue (wet wt) Intestine	Liver	Liver molybdenum content (dry wt basis), ppm	Ratio of liver enzyme activity to molybdenum content
None	1.58	459.7		54.7	.240	40.4	70.9	2.54	27.9
6% distillers dried solubles	1.68	528.0	14.9†	56.4	.216	47.3‡	99.7†	2.96	25.4
.0254 ppm molybdenum (as molybdic acid)	1.61	526.0	14.4†	56.6	.444	56.2‡	94.0†	3.15	26.7
Distillers dried solubles ash	1.68	588.5	28.0‡	57.0	.312	52.3‡	92.6†	3.20	25.4
U. G. F. mixture*	1.98	666.0	44.9‡	56.7	.374	64.5‡	140.7‡	5.95	23.6
L. S. D. .05	—	65.5	14.2			4.7	21.5		
.01	—	86.7	18.9			6.4	29.4		

* 3% distillers dried solubles, 6% fish solubles, 6% dried whey, 3% antibiotic fermentation residue, and 3% forage juice.
† Significant at .05 level of probability over unsupplemented group.
‡ " " .01 " " " " " .

tation residue, and forage juice produced a 44.9% increase in growth ($P<0.01$). It is suggested from these data that a portion of the unidentified factor response may be explained on the basis of molybdenum in the factor sources.

Slight increases in bone ash values were obtained with supplemented diets. A 1.7% increase in bone ash was obtained with 6% distillers dried solubles, 1.9% increase with molybdenum, 2.3% with distillers dried solubles ash, and 2% with the unidentified factor mixture. Morrison *et al.*(12) have reported significantly higher ($P<0.02$) bone ash values in chicks upon feeding of ash of a mixture of distillers dried solubles, dried whey, fish solubles, forage juice and penicillin mycelia meal; bone ash was increased from 45.07% for the basal group to 47.30% for the supplemented group in this report.

Molybdenum determinations on bone ash of turkey poults demonstrated slightly more of the element present in ash from birds fed molybdic acid, distillers dried solubles ash or the U.G.F. mixture as compared to the basal group. There was slightly less molybdenum present in the bone ash of 6% distillers dried solubles fed poults (Table I) than in control birds.

Intestinal xanthine dehydrogenase activities, determined using hypoxanthine as substrate with methylene blue as electron acceptor, were increased significantly ($P<0.01$) over that of the control group by all supplements tested. Enzyme activity exhibited by the group fed 0.0254 ppm added molybdenum as molybdic acid was significantly greater ($P<0.01$) than that obtained with the distillers dried solubles group (Table I). The ash of distillers dried solubles was more active ($P<0.05$) in stimulating intestinal xanthine dehydrogenase activity than the untreated product, but was not significantly different from molybdenum in this regard (Table I).

The mixture of supplements was more effective than any of the supplements tested in eliciting a response in intestinal xanthine dehydrogenase. These data again suggest that a portion of molybdenum in distillers dried solubles was unavailable to the poult, since one-fourth the level of molybdenum in distillers dried solubles added as molybdic acid was significantly more effective than was distillers dried solubles. Ash of distillers dried solubles, although more active in this respect than the unashed product, was no more active than was molybdenum fed at a level of one-fourth that supplied in the ash.

Liver xanthine dehydrogenase values showed significant increases ($P<0.05$) due to addition of either distillers dried solubles, molybdenum (0.0254 ppm) or distillers dried solubles ash to the basal diet (Table I). However, no significant differences in liver enzyme activities between these 2 supplements were obtained. A highly significant response in enzyme activity was obtained with the mixture. The liver molybdenum contents corresponded quite well with level of enzyme activity exhibited. A correlation coefficient of 0.955 was obtained which proved significant at the 0.05 level of probability. The ratio of enzyme activity to molybdenum content of liver was also fairly constant over levels of the element determined, with the average for all groups at 25.8.

Summary. (1) Weights of turkey poults fed a purified type diet were increased significantly upon addition of distillers dried solubles, distillers dried solubles ash, molybdenum (0.0254 ppm) or a mixture of distillers dried solubles, dried whey, fish solubles, an antibiotic fermentation residue, and forage juice. Although differences of as much as 2% in bone ash were observed, statistical significance was not obtained. (2) Both liver and intestinal xanthine dehydrogenase activities were significantly higher in supplemented groups as compared to control birds. A significant ($P<0.05$) correlation was determined between liver molybdenum content and level of enzyme activity. (3) Under the conditions of this study a portion of growth promoting activity of distillers dried solubles ash would appear to be explained on the basis of the molybdenum content.

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Isolation and Identification of Infectious Bovine Rhinotracheitis Virus in Tissue Culture. (23071)

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A disease of cattle characterized by severe inflammation of upper respiratory passages and trachea, accompanied by excessive nasal discharge, salivation, dyspnea and fever has been reported since 1950 in about 15 western states(1,2,3). This condition, observed in both dairy and beef cattle areas, has been described under various names such as acute upper respiratory infection, infectious necrotic rhinotracheitis, or more commonly, red nose disease. At a meeting of the U. S. Livestock Sanitary Assn. in Nov. 1955, it was generally agreed to call this infectious bovine rhinotracheitis (IBR). The disease could be reproduced regularly by intranasal inoculations of cattle with nasal washings from naturally infected cases, and protection tests in cattle, using infectious material from different parts of the country, indicated that the illness was caused by the same etiological agent(4,5). However, attempts to isolate this agent in chick embryos, weanling and suckling mice, guinea pigs and rabbits were unsuccessful(5). In a preliminary report, it was shown that with the use of bovine embryonic tissues in tissue culture, a virus producing a cytopathogenic effect on cells was isolated from infectious material of cattle(6). This paper presents additional information concerning this virus, and shows that it is the etiological agent of infectious bovine rhinotracheitis.

Material and methods. Tissue culture procedures. The cortex of bovine kidneys obtained from 8-9 months old feti was minced and trypsinized by a method similar to that described by Youngner(6). The prepared cells were suspended in nutrient medium consisting of 0.5% lactalbumin hydrolysate, and 5% to 10% horse serum made up in Earles basic salt solution. To this was added 200 units of penicillin and 200 μg of streptomycin/ml. This mixture was then dispensed in 2 ml amounts in tubes and allowed to stand in a stationary position for 5 days, or until good cellular growth was observed. At this time, the medium was changed and tubes placed in roller drums for use. Similar preparations were made with bovine testicular tissue. Tubes made with lung tissue, however, were prepared by a plasma-clot technic employing small pieces of minced tissue(7). *Sources of infectious material.* Various tissues, as well as nasal washings, were obtained during the early acute phase of illness from cattle naturally infected with IBR disease in Colorado, California, and Ohio. These specimens were placed in screw-cap vials and frozen immediately in an alcohol dry-ice bath, and stored at

TABLE I. Isolation of IBR Virus from Tissues of Naturally Infected Cattle from Different Areas.

Source	Specimen	Isolations/ Attempts	Year	Strain designation
Colorado beef	Turbinate	6/6	1955	Colorado I & II
	Larynx	2/2		
	Trachea	3/5		
	Nasal washing	3/3	1955–56	" III, IV, V
	Spleen	0/5	1955	
	Liver	0/4		
	Lung	0/7		
California beef	Nasal washing	1/1	1955	Blythe
" dairy	" "	1/1	1955	Los Angeles (L.A.)
Ohio beef	Turbinate	1/1	1956	Ohio I

–70°C until use.

Results. 1. *Isolation of virus.* Tissue cultures showing a continuous sheet of epithelial cells of bovine embryo kidney on the sides of tubes were selected for inoculation with infectious material. Portions of turbinate, larynx, trachea, lung, liver, and spleen from infected cattle were made into 10% suspensions in tissue culture medium, using a glass tissue grinder, and centrifuged at 1,000 rpm for 10 minutes. Supernatants of each suspension were inoculated in 0.2 ml amounts into each of several tubes. Similar tubes were inoculated with 0.2 ml of undiluted nasal washings. These tubes were observed daily for at least 7 days, following which transfers of 0.2 ml quantities of tissue culture fluid were made into new tubes of tissue culture cells. After a sub-culture was made and no demonstrable changes occurred, the tubes were discarded. A summary of various isolation attempts is presented in Table I.

Two to 3 days after inoculation with infectious material, a cytopathogenic effect (CPE) was observed. The cells became more granular, then rounded up and refractile in appearance. The affected cells grouped together to form clumps and strands. This effect was progressive until all cells became involved and were finally released from the wall of the tube. Similar cytological changes occurred when tissue cultures of testicular and lung tissues were inoculated with infectious material. The general appearance of this cytological change remained the same in all passages, although the rapidity with which all cells in the tube became affected depended on the titer of virus inoculated. A comparison of normal and infected bovine embryonic kidney epithelial tissue culture cells can be seen in Fig. 1.

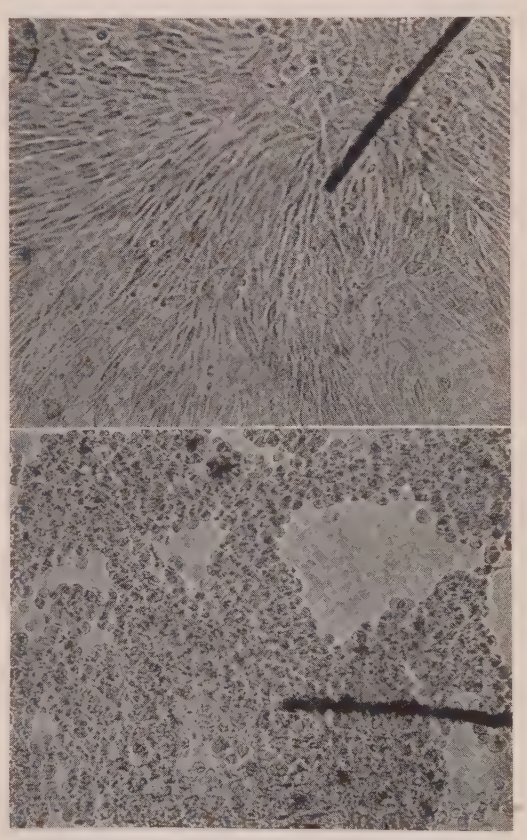

FIG. 1. Comparison of normal bovine kidney tissue (above) with a similar culture (below), showing complete cytopathogenic effect following inoculation with the virus of infectious bovine rhinotracheitis.

Titrations of infected kidney tissue culture fluids were made of a number of passages, using the CPE as an indicator of end point. By preparing 10-fold serial dilutions of the 8th tissue culture passage of 1 strain of virus (Colorado I) and inoculating 0.2 ml into each of 4 tubes for each dilution, a 50% end point ($TCID_{50}$) of infectivity was obtained at $10^{-6.5}$. This would indicate that the virus had multiplied in the tissue culture, since the end point of titration for the 8th passage would represent a dilution of $10^{-14.5}$ of the original tissue suspension. Subsequent titrations through 100 passages of this virus gave similar results with $TCID_{50}$ end points varying from $10^{-5.5}$ to $10^{-7.0}$.

2. *Studies with cattle.* (a) Reproduction of the Disease. All cattle used in these studies were either raised on our research farm or purchased from the area immediately surrounding the laboratory (a region where the disease has not yet been reported). The animals varied from 4-9 months of age, and were generally held in isolation for at least 2 weeks prior to inoculation. Daily temperature records, as well as other observations, were made to insure that animals were normal before use.

To determine whether the virus causing the CPE in tissue culture produced IBR disease in experimental cattle, groups of animals were inoculated intranasally with 1 ml of infected tissue culture fluid, using different tissue culture passage levels of the Colorado I strain. The calves were inoculated as follows: 4 with T.C. passage 2; 9 with T.C. passage 4; and 6 with T.C. passage 15. Within 2-3 days after inoculation, a febrile response occurred in each inoculated animal varying from 103.6° to 106.6°F and lasting from 1-9 days, with average duration of 3½ days. Other signs of illness, such as nasal discharge, depression, inappetence, dyspnea, and coughing were also noted, although not all of these were observed in each experimental animal. A high percentage of animals showed marked hyperemia of the nasal mucosa as well as small, white areas of serofibrinous exudate which adhered tightly to the anterior portion of mucosal surfaces. This condition generally persisted a few days after disappearance of the febrile response. In more severe cases, this serofibrinous exudate became extensive and covered not only anterior areas of the nasal mucosa, but also extended posteriorly over the surfaces of turbinates. There appeared to be no discernible difference in response of the cattle to different passage levels of tissue culture material tested. Several animals, inoculated with normal tissue culture fluid, remained healthy and susceptible to challenge with virulent virus 2-3 weeks later.

TABLE II. Cross-Protection Tests in Cattle with Several Strains of Infectious Bovine Rhinotracheitis Virus.

Immunizing strain	Challenging strain			
	Col. I T.C.*	Col. V N.W.†	Col. V T.C.	Blythe T.C.
Colorado IV N.W.	0/ 1‡	N.T.§	N.T.	N.T.
" I T.C.	0/20	1/11	0/3	0/3
Blythe T.C.	0/ 1	N.T.	N.T.	N.T.

* T.C. = Tissue culture-propagated virus.
† N.W. = Nasal washings from naturally infected cattle.
‡ Numerator = No. of animals reacting to challenge; denominator = No. of animals challenged.
§ N.T. = Not tested.

(b) Identification by protection tests. To determine whether the experimentally produced disease was actually infectious bovine rhinotracheitis, 39 calves were immunized with one or another virus strain. They were challenged intranasally 2-3 weeks later with homologous or heterologous virus shown to have disease-producing properties by inoculation of one or more susceptible control animals. The results are summarized in Table II.

3. *Identification by serum neutralization test.* As further proof that the cytopathogenic agent isolated from infectious material was the cause of IBR, serum neutralization tests were undertaken. In these tests, equal amounts of serum, or serum dilutions, were mixed with equal amounts of infected tissue culture fluid in a final concentration between 100 to 1,000 $TCID_{50}$ of virus. This mixture was incubated for 2 hours at 37°C and 0.2 ml then inoculated into each of 3-4 kidney tissue culture tubes and observed for several days. The serum neutralization effect was

TABLE III. Reciprocal Cross-Neutralization Tests with 3 Strains of Infectious Bovine Rhinotracheitis Virus, Using Homologous and Heterologous Immune Sera against 32-200 $TCID_{50}$ of Virus.

Sera tested		Strains of virus used		
		Colorado 1	Blythe	L.A.
Colorado	1	1: 10	1: 9	1: 9
	2	1:180	1:100	1:180
	3	1: 6	1: 3	1: 3
Blythe	1	1: 10	1: 19	1: 10
	2	1: 45	1: 45	1: 10
	3	1: 27	1: 27	1: 23
	4	1: 13	1: 10	1: 10
L.A.	1	1:180	1:180	1:180
Normal serum*		0	0	0

* Normal serum represents 3 tests with pre-inoculation sera from cattle inoculated with Colorado 1 virus.

recorded when approximately 100 $TCID_{50}$ units of virus were observed in the virus control titration carried out simultaneously.

By this method, it was demonstrated that the animals inoculated with Colorado I developed antibodies against this strain in a titer ranging from 1:2 to 1:64. Uninoculated control animals, or animals inoculated with normal tissue culture fluid, were negative for neutralizing antibodies against this virus. Serum samples collected from 71 animals which had recovered from IBR during natural outbreaks in California, Colorado, Illinois, Nebraska, and Ohio had neutralizing antibodies against Colorado I. Colorado V, Blythe, L. A., and Ohio tissue culture isolates were also neutralized by Colorado I antiserum.

To examine more completely the antigenic relationship between these viruses, 3 strains obtained from widely separated areas (Colorado I, Blythe, and L.A.) were used in reciprocal cross-neutralization tests employing antisera against each of these viruses. The results of this work, summarized in Table III, indicate a close, if not identical, antigenic relationship between these strains of virus.

Discussion. The evidence presented seems to leave little doubt that the tissue culture virus is the etiological agent of IBR disease. A significant fact is that this virus produced illness in experimental cattle characteristic of that observed in naturally occurring cases. It is possible, however, that similar upper respiratory infections might be caused by a number of agents. Therefore, additional support for the relationship of this virus to the disease is provided by protection tests in cattle. If the tissue culture virus was not the etiological agent, then animals immunized with this virus should have responded to a challenge inoculum of IBR infectious material obtained from field outbreaks of the disease. As has been shown, this did not occur. Final proof as to the identity of the virus is provided by the fact that antibodies produced by the first tissue culture isolate neutralized equally well its homologous virus and viruses isolated from infectious material from different IBR outbreaks. Conversely, sera from these outbreaks readily neutralized the tissue culture virus.

Production of a cytopathogenic effect in tissue culture cells by this virus provides an easy and accurate means of measuring the titer of infectious material. It also furnishes a method for additional isolations of the virus from various outbreaks of this disease. The neutralization test in tissue culture makes possible a serological test that can be used to study the epidemiology of the disease in individual herds or areas, as well as to identify new outbreaks of the infection that may occur in different parts of the country.

Since bovine embryonic tissues are readily available, growth of this cytopathogenic virus in tissue culture is a major step forward in propagating the virus in quantity for possible use in development of a practical vaccine. Furthermore, the fact that all strains of virus so far isolated appear to be antigenically the same greatly simplifies the task of developing an effective control program.

Summary. An agent producing a cytopathogenic effect has been isolated in bovine embryo tissue culture cells from upper respiratory tissues of cattle having the disease of infectious bovine rhinotracheitis. Lack of growth in bacteriological culture media, resistance to antibiotics such as penicillin and streptomycin, and ability to pass through fine porosity filters demonstrates that this agent is a virus. Reproduction of a disease in experimental cattle identical to that which occurs naturally, protection tests in cattle using

tissue culture virus and infectious material from naturally occurring cases, as well as neutralization tests with serum obtained from naturally and experimentally infected cattle, show that this virus is the etiological agent of infectious bovine rhinotracheitis. Serum neutralization tests also indicate that viruses isolated from different parts of the country are closely related antigenically.

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Increased Production and Utilization of Circulating Glucose During Growth Hormone Regimen.*† (23072)

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Hypophysectomized dogs exhibit extensive abnormalities in carbohydrate metabolism (1), many of which are reversed by growth hormone(2). Large doses of the hormone may produce diabetes in the normal dog(3). Recently, by application of a technic whereby body glucose pool was tagged with C^{14}(4), it was shown that the unanesthetized hypophysectomized dog in postabsorptive state, in comparison with the normal dog, had a smaller body glucose pool and lower rate of glucose turnover, *i.e.*, glucose inflow from liver into plasma, and glucose outflow from plasma into tissues(5).

The present study is concerned with effect of a growth hormone regimen on turnover rate of the glucose pool in the unanesthetized hypophysectomized dog, studied with the aid of C^{14} glucose.

Materials and methods. Adult hypophysectomized male and female dogs, maintained on standard diet(2), were used. The animals were 57-850 days postoperative. Growth hormone (Lot C_5I_5; prepared and kindly supplied by Dr. Robert Bates) was administered intramuscularly in daily doses of 1 mg/kg for 4-5 days, last injection given 18 hours prior to experiment. The animals were in good physical and nutritional state before and during injection period. The experiments were performed after 17-18 hours fast and without anesthesia. A complete description of experimental material and procedure has been given previously(4). Uniformly labeled C^{14} glucose was administered intravenously by initial priming injection, followed immediately by constant continuous infusion. Total amount of glucose administered did not exceed 4 mg. Samples of blood were drawn at intervals for determination of concentration of plasma glucose and its C^{14} content. Plasma glucose content was determined on aliquots of Somogyi zinc-barium filtrates(6), by the method of Hagedorn-Jensen(7). Unlabeled carrier glucose was then added to other aliquots of the filtrate and glucose was

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TABLE I. Plasma Glucose Concentration and Glucose Inflow-Outflow Rates in Normal, Hypophysectomized and Growth Hormone-Treated Hypophysectomized Dogs.

Dog status	Avg plasma glucose, mg %		Glucose inflow-outflow, g/m²/hr
Normal (10)*	107		3.80 ± .19†
Hypophysectomized (7)	89		2.59 ± .27
Hypophysectomized on growth hormone (1 mg/kg, 4-5 days)			
AL-22	95‡	4.07	
AL- 3	134	3.76	3.89 ± .09
AL-16	109	3.85	

* No. of experiments.
† Mean and stand. error of mean.
‡ Compared with 81 mg prior to growth hormone treatment.

isolated as the phenylosazone derivative. Rate of glucose turnover was calculated as described previously(4).

Results. Table I gives average plasma glucose concentration and turnover rate of body glucose in normal, hypophysectomized and growth hormone-treated hypophysectomized dogs. Composite values for normal and hypophysectomized dogs were obtained in earlier studies(5). The turnover rate is presented as grams glucose/m² body surface area/hr. Surface area was calculated according to Rhoads, Alving, Hiller and Van Slyke(8), using the weight at time of experiment. The outstanding observation in this study was the increase in rate of glucose inflow-outflow during the growth hormone regimen. Thus more glucose was released by the liver into the plasma and more glucose disappeared from the plasma into the tissues.

Growth hormone treatment in hypophysectomized dogs also raised plasma glucose concentration to normal or above. Although the rise is less apparent for dog AL-22, it should be noted that hormone treatment elevated the glucose concentration 14 mg% above the control. The effect of growth hormone on glucose pool size remains to be clarified.

Discussion. The present study demonstrates that growth hormone regimen resulted in an increased release of glucose from the liver and an increased uptake of plasma glucose by tissues. This was observed during growth hormone treatment in normal and adrenalectomized dogs as well as in hypophysectomized dogs. In hypophysectomized dogs, as shown here, growth hormone treatment restored to normal their low rate of glucose inflow-outflow(5).

The ability of growth hormone to increase glucose inflow from the liver is even more striking when considered together with the results obtained following insulin injection in untreated hypophysectomized animals(9). Thus when an intravenous injection of insulin (glucagon-free) increases glucose uptake by the tissues, the liver of the hypophysectomized dog, in contrast to the normal one, is unable to increase its glucose output, and consequently a prolonged hypoglycemia is observed. During growth hormone regimen, as shown in the present experiments, increased uptake of glucose during postabsorptive state was associated with increased glucose release from the liver. These findings stress the central position of the liver in the growth hormone action. A similar increased glucose release by the liver was obtained with hydrocortisone or cortisone(10).‡ However, whereas in steroid-treated animals the additional glucose is probably of protein origin, as evidenced by an increased excretion of urinary nitrogen, the growth hormone-induced increase in glucose release occurs at a time of diminished nitrogen excretion(11), increased fat mobilization(12) and while the liver glycogen is not effectively mobilizable by epinephrine(13).

The question may be raised whether the increased glucose utilization is associated with an increased secretion of insulin. Indeed, Randle(14) observed an increased plasma insulin activity in growth hormone-treated animals, even during the temporary diabetic state. The enhanced glucose utilization and increased plasma insulin activity during growth hormone treatment are in harmony with the findings that growth hormone-induced nitrogen retention is dependent upon adequate glucose utilization(15). The present data suggest that the disappearance of insulin hypersensitivity(2), when a hypophy-

‡ Reported in *Fed. Proc.*, 1956, 3.

sectomized dog is kept on a growth hormone regimen, involves other factors besides direct growth hormone inhibition of insulin action.

Summary. Using C^{14} glucose to label the body glucose pool, it was found that growth hormone treatment for 4-5 days in the hypophysectomized dog, resulted in increased rate of glucose release by the liver and plasma glucose uptake by the tissues. The plasma glucose concentrations were also increased. The significance of these findings is discussed.

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Effects of Prolactin on Duration of Pregnancy, Viability of Young and Lactation in Rats. (23073)

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Several investigators have demonstrated that prolactin is luteotrophic in the rat. Evans *et al.*(1) reported that prolactin was the only anterior pituitary hormone capable of stimulating luteal function in hypophysectomized rats, while Desclin(2) and Mayer and Canivenc(3) found that it prolonged luteal function in normal cycling rats. Cutuly(4) showed that prolactin maintained early pregnancy in rats mated and then hypophysectomized, either before or after implantation of the ovum. However, Mayer and Klein(5,6) reported that prolactin failed to prolong gestation in intact rats when injected beginning on the 14th or 19th day of pregnancy, and continuing until or beyond the normal day of parturition. They also found that prolactin did not prolong the life of corpora lutea of pregnant rats following removal of uterine horns, presumably because the luteotrophic influence of the placentae was removed. They concluded that pituitary prolactin does not exert a luteotrophic action in rats during the latter half of pregnancy.

The present experiments were undertaken to determine the effects of several levels of purified prolactin on duration of gestation, functional activity of corpora lutea and initiation of lactation in rats. In general, the results show that the prolactin preparation used by us prolonged pregnancy and maintained functional activity of the corpora lutea, without preventing initiation of lactation.

Methods. One hundred and twenty-four

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TABLE I. Effects of Prolactin on Duration of Pregnancy and Viability of Young in Rats.

Group and No. of rats	Treatment	Day of parturition					Avg No. of young		Litters retained in uterus
		21	22	23	24	25	Alive	Still-born	
(1) 39	Controls, no treatment	32	7				8.5	.3	0
(2) 10	.25 mg prolactin daily 16-20th day of preg.		9				7.9	.0	1
(3) 10	*Idem* 25th	1	5	3			6.2	.3	1
(4) 15	.5 mg prolactin daily 16-20th day of preg.		6	3	4		3.7	2.4	2
(5) 5	*Idem* 25th		3				3.8	1.0	2
(6) 10	1 mg prolactin daily 16-20th day of preg.	3	1	4			2.8	1.9	2
(7) 10	*Idem* 25th		1		2	2	1.0	1.0	5
(8) 5	4 mg prolactin daily 16-20th day of preg.						.0	.0	5
(9) 5	1 mg prolactin daily 16-20th day of preg. Ovariectomy 19th day preg.	5					6.0	1.2	0
(10) 5	1 mg prolactin daily 16-20th day of preg. Ovariectomy 20th day preg.		5				7.1	.7	0
(11) 10	7.5 mg progesterone daily 16-20th day of preg.			1	4	5	.0	8.5	0

pregnant albino rats, originally of Wistar strain, were used. They weighed from 165 to 220 g each, and were housed in air-conditioned quarters with Purina laboratory chow and drinking water available at all times. The day sperm were found in the vagina was considered the first day of pregnancy. With the exception of 4 groups of untreated pregnant rats, which served as controls, and one which received progesterone‡ all other groups were injected subcutaneously with .25, .50, 1.0 or 4.0 mg of prolactin§ daily from 16th through 20th days of pregnancy. In 3 groups, prolactin injections were continued through 25th day. The day of parturition was recorded for each rat, as well as the number of young born alive, stillborn or dead *in utero*. Attempts were made to express milk from the nipples beginning the 21st day. At the end of 26 days, all rats which had not dropped their young were killed and mammary glands and ovaries removed for gross and histological examination.

Results. Thirty-two of 39 control rats (Group 1) dropped their young on 21st day, and 7 on 22nd day. An average of 8.5 young were born alive and 0.3 young were born dead to each mother. When 0.25 mg of prolactin was injected from the 16th to 20th day of gestation (Group 2), 9 of 10 rats were delivered of offspring on the 22nd day and one failed to be delivered even by the 25th day. The same daily amount of prolactin given through the 25th day (Group 3) extended the average duration of pregnancy, and fewer living young were born per rat (Table I).

Injections of 0.5 mg (Groups 4 and 5) or 1 mg of prolactin daily (Groups 6 and 7) further prolonged gestation and increased the number born dead or retained *in utero*. Treatment with prolactin to the 25th day was more effective in these respects than when terminated on the 20th day. When 4 mg of prolactin was injected (Group 8), none of the rats was delivered of young by 26th day and all fetuses died *in utero*. Prolongation of gestation beyond the 23rd day resulted in death of most young.

Groups 9 and 10 were ovariectomized on the 19th or 20th day of gestation after receiving 1 mg of prolactin daily from 16th through 20th day of pregnancy. These rats were delivered 2 days after ovariectomy and most of the young were born alive. When 7.5 mg of progesterone was injected from 16th through 20th day of pregnancy (Group

‡ The crystalline progesterone was provided through the courtesy of Dr. J. O. Reed of Foundation Laboratories, New York.

§ We are indebted to Dr. A. Borman of Endocrine Research Section, Squibb Institute, New Brunswick, N. J., for prolactin, which was stated to contain approximately 15 I.U./mg.

11), the time of parturition was delayed and all young were born dead.

Secretion by mammary glands was initiated in almost all mother rats by the 21st day, regardless of how much prolactin they were given, but the amount of lactation appeared to be less in mothers with extended pregnancy than in those whose pregnancy was terminated. The corpora lutea of ovaries of prolactin-treated rats were large, well vascularized and were markedly predominant over follicular cells.

Discussion. Results of these experiments are believed to demonstrate that prolactin extends pregnancy in rats under our conditions, and that this depends upon dose of hormone and duration of treatment. The largest dose, 4 mg daily, was most effective in inhibiting parturition, while .25 mg daily had almost no effect on duration of pregnancy. When the period of gestation was extended by prolactin, fewer young were born alive and a greater number were born dead or retained *in utero.*

Since prolactin failed to prolong pregnancy in rats which had their ovaries removed just prior to expected parturition, it is clear that the pituitary hormone acts to extend gestation via its stimulation of the ovary. Moreover, since it has been demonstrated that injections of crystalline progesterone will extend gestation (Group 11), it appears that the prolactin action is to stimulate corpora lutea and increase secretion of systemic progesterone. Histological examination of the ovaries revealed large and extensive corpora lutea.

Lactation was initiated in prolactin-treated rats about the 21st day after fertilization, regardless of whether young were expelled or retained in the uterus. However, degree of secretion was usually greater in mammary glands of mothers which dropped their young than in those which retained them. This supports the view that antagonism between mammary growth and lactation during pregnancy is relative and both can proceed simultaneously(7,8). Earlier workers had reported that injections of crude prolactin extracts into pregnant rats resulted in initiation of lactation only after abortion or resorption of the embryos(9).

It is difficult to account for differences between results reported here and those of Mayer and Klein(5,6). They injected rats with 30 to 90 I.U. of prolactin daily for 5 to 10 days beginning on the 14th or 19th day of pregnancy, while we injected approximately 3.7 to 60 I.U. of prolactin daily for 5 to 10 days beginning on the 16th day of gestation. It is possible that factors other than prolactin in the preparations used influenced the results. The exact nature of the factor in rat placentae which can maintain gestation in hypophysectomized rats after midpregnancy is unknown, but Canivenc and Mayer (10) suggested that it is identical biologically with prolactin since it induced proliferation of the pigeon crop gland.

Summary. 1. Injections of approximately 3.7 to 60 I.U. of prolactin daily for 5 to 10 days, beginning on the 16th day of gestation, prolonged pregnancy in rats. The largest doses and longer treatment were most effective in preventing parturition, resulting in death of most of the young when pregnancy was extended beyond 23 days. 2. Prolactin failed to prolong pregnancy in rats ovariectomized on the 19th or 20th day of pregnancy, while injections of progesterone into intact rats extended pregnancy. These results as well as histological examination of corpora lutea, suggest that prolactin acts by extending functional activity of corpora lutea of pregnancy. 3. Lactation was usually initiated in prolactin-injected rats by the 21st day of gestation, independent of the occurrence of parturition. However, milk secretion was not as marked in rats which failed to be delivered of their young as in parturient rats.

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Effects of Hyaluronidase and Cortisone on Connective Tissue Studied Electrometrically.* (23074)

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The ground substance of connective tissue behaves in many respects as heterogeneous colloid consisting of negatively charged aggregates. Of these, mucoproteins form a prominent component(1). The physico-chemical state of the ground substance is modulated by variations in internal environment and through reversible action of certain hormones and enzymes(2-5). An example of reversible behavior, reported in this paper, is the resynthesis of connective tissue colloids following disaggregation induced by hyaluronidase.

Many of the properties of the ground substance have been related to colloidal charge density, which can be determined electrometrically by measurement of liquid junction potentials(1-3). The electrometric method was used in this investigation to study disaggregation and reformation of connective tissue colloids in normal and cortisone treated rabbits after injection of hyaluronidase. The rate at which this occurs is believed to reflect a synthetic activity of cells. The results suggest that this procedure may be useful as a test of biologic resiliency of connective tissue.

Material and methods. Liquid junction potentials were measured serially on 22 albino rabbits, approximately 3 months of age. Eight of these animals received two 5 mg intramuscular injections of cortisone acetate (Merck) 48 and 24 hours prior to time of analysis. In 4 of these rabbits, electrometric determinations were also made before cortisone was administered. The animals were anesthetized by an intraperitoneal injection of 3% sodium pentobarbital. A 20-gauge one-half inch hypodermic needle was then inserted into the dermal layer of the shaved anterior abdominal wall. A reference junction was formed by placing a similar needle under the skin of the left hind leg.† After the desired salt solutions were instilled, the needles were connected to small syringe barrels filled with saturated KCl in agar which, in turn, were joined by saturated KCl bridges to calomel half cells. A Leeds-Northrop type K-2 potentiometer and galvanometer completed the circuit, which may be represented as follows:

Hg	Hg_2Cl_2	KCl (sat.)	NaCl (.15 M)	Subcut. tissue (refer.)	—

—	Subcut. tissue (test site)	NaCl (I or II)	KCl (sat.)	Hg_2Cl_2	Hg

A baseline potential (E) was measured with 0.15 M NaCl (solution I) at both junctions. In each instance final value was based on the average of 3 consecutive readings taken at approximately 30-second intervals, and agreeing within one millivolt. The isotonic saline was then withdrawn from the experimental needle (abdomen) by aspiration with a 27-gauge needle, replaced by solution II, a 1/10

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† The reference junction may also be conveniently made by placing the shaved ear in a beaker of isotonic NaCl.

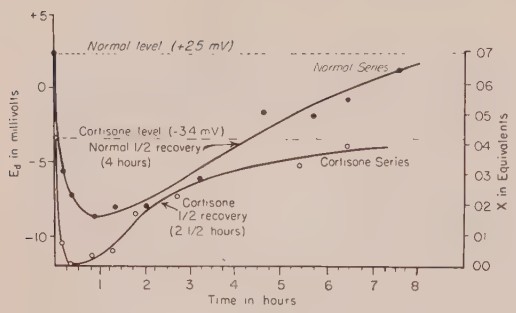

FIG. 1. Effect of hyaluronidase on dermal connective tissue in normal and cortisone treated rabbits. Each experimental point is mean of 5 or more observations. Standard deviations ranged from 1.0 to 2.4 in normal animals, and 0.5 to 1.7 in those receiving cortisone. Half recovery time in each series is estimated from mid-point between initial level and point of maximal displacement.

dilution (0.015 M NaCl), and the displaced potential (E′) determined. Finally the dilute salt solution was replaced with 0.15 M NaCl, and the original baseline was restored. The difference, E′-E, is termed the dilution potential (E_d). After measurement of initial dilution potential, 5 turbidity reducing units (TRU) of a freshly prepared solution of testicular hyaluronidase‡ dissolved in 0.1 cc of 0.15 M saline was injected at the test site. Dilution potentials were determined at frequent intervals at first and later at more widely spaced times until the values approached their initial levels (Fig. 1). The relationship between the dilution potential and the density of immobile negative charge in the connective tissue (x) can be expressed by the approximation formula: $E_d = -12.3 + 215\ x$, where E_d is measured in millivolts and x is expressed as equivalents/kg of tissue water(1,5,6).

Results. The results of dilution potential determinations in the dermis of normal and cortisone treated animals are compared in Fig. 1. The average dilution potential in the normal rabbit was $+2.5 \pm 1.0$ mv corresponding to a colloidal charge density of 0.069 equivalents.§ In animals pretreated with cortisone, the initial dilution potential averaged -3.4 ± 1.3 mv corresponding to a colloid charge density of 0.041 equivalents, or 60% of the normal level. After hyaluronidase action, dilution potentials decreased on the average to –8.5 mv for normal animals, and to –12.0 mv for cortisone treated rabbits. Return of dilution potentials to the initial state occurred more rapidly in the cortisone series. Half-recovery in this group required an average of about 2½ hours as compared with 4 hours for normal animals.

Discussion. Continuous changes in the state of connective tissue can be studied by measuring dilution potentials as a function of time. The relation of such measurements to the concentration of negatively charged colloid has been established(5,6). In dense connective tissue such as bone and cartilage, where the colloid is considered to be highly aggregated, the dilution potential may attain values of +25 mv and x may be as high as 0.175 equivalents/kg of tissue water. In loose tissue, as for example synovial fluid, the values of E_d tend to approach the theoretical limit of –12.3 mv for the state where x approaches zero.

Certain hormones and enzymes alter the state of connective tissue colloids, increasing fraction of soluble colloids and lowering density of immobile negative charge. Dilution potentials may then tend to approach the lower theoretical limit. When these effects are reversed, potentials return to their initial levels. Accordingly, electrometric readings over an extended period of time offer an objective means of studying reversible responses of connective tissue.

Electrometric studies of rabbit skin indicate that normally the colloids of the dermis are not highly aggregated. Treatment with cortisone produces a decrease in E_d. As a result of this initial modification of connective tissue the effect of hyaluronidase in the cortisone treated animals is enhanced. Previous histochemical and electrochemical studies have shown that cortisone administration in the rat leads to the formation of a

‡ Wyeth and Co. contributed a generous supply of Wydase.

§ Experiments in which liquid junctions are made directly to exposed dermis by means of moistened cotton have confirmed the existence of contiguous regions showing dilution potentials between -2 and +2 millivolts.

more loosely aggregated ground substance in the skin(3). Cortisone appears to increase the rate of formation of certain connective tissue colloids. However, these components have modified physicochemical properties. Stimulating effects of cortisone upon connective tissue have also been demonstrated in nasal mucosa altered by the allergic state(7) and in atopic dermatitis(8).

The rate at which connective tissue is disaggregated and restored following administration of hyaluronidase may be used as an index of its initial state and of its capacity for repair. It is probable that this response varies under the influence of many other hormones and drugs, as well as in disease states. The serial electrometric determination of these changes in connective tissue colloids may serve as a connective tissue "function" test, measuring the ability to maintain homeostasis.

Summary. Reversal of hyaluronidase effects on concentration of negatively charged colloids in the dermis of normal and cortisone treated rabbits was studied by serial measurement of liquid junction potentials. In normal animals, hyaluronidase lowers negative charge density in the tissues; recovery is half completed in about 4 hours. In animals treated with cortisone initial charge density is lower than in normal animals. After hyaluronidase is injected into the dermis, the lowest levels are reached. Half recovery, however, occurs in 2½ hours. Serial electrometric measurements following hyaluronidase injection are suggested as a means of studying hormone and drug effects, and disease states in connective tissue.

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Lack of Specificity of Insulin-I^{131}-Binding by Isolated Rat Diaphragm. (23075)

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Stadie and coworkers reported an increase in glycogen synthesis by the isolated rat diaphragm exposed briefly to solutions of insulin(1). It was suggested that insulin is bound firmly to tissues at or near sites of enzyme systems and that such fixation is necessary for its physiologic action. Stadie, Haugaard and Vaughn(2) later noted that, following brief immersion of diaphragm into solutions of S^{35}-labeled or I^{131}-labeled insulin, a small fraction of radioactivity remained fixed to the tissue even after repeated washing with saline solution, and with increasing concentrations of insulin there was a tendency for bound insulin to reach a limiting value at about 1½ to 2 μg/g tissue.

In the course of studies with a variety of I^{131} labeled proteins from different sources, *e.g.*, serum albumin, gamma globulin, insulin, glucagon, ACTH, diphtheria toxoid, etc., we observed that, at tracer levels, most of these proteins adsorb nonspecifically to almost any inert surface, and that such adsorption may be minimized by presence of high concentrations of almost any other protein. With insulin, particularly, adsorption to glass(3), and paper(4,5) has been reported previously. It therefore seemed of interest to reevaluate

the specificity of the binding of isotopically labeled insulin to diaphragm *in vitro*.

Methods. I^{131} labeled insulin was prepared from crystalline beef insulin* with specific activity of 1-3 mc/mg by modification of the method of Pressman and Eisen(6). This modification consists of extraction with chloroform of elemental iodine produced in Pressman-Eisen procedure and employment of $CHCl_3$-I_2 solution for iodination of proteins. By this procedure the yield of protein-bound I^{131} is increased to about 30% of the starting radioactivity and the iodinated proteins appear to preserve their biologic integrity(5). All preparations were dialyzed for 18-24 hours until nonprotein bound I^{131} was less than 1% of total radioactivity. I^{131} labeled pooled human serum albumin and gamma globulin were prepared in a similar manner from commercially available sources of these proteins and I^{131} labeled glucagon was prepared from crystalline glucagon.† Assays of radioactivity were performed in a well-type scintillation counter with sensitivity of 1 x 10^6 c/m/μc I^{131} above a background of 200 c/m. Sufficient counts were made in all experiments to reduce the statistical error to less than 3%. The general procedure for determination of insulin-I^{131} bound to diaphragm was essentially the same as that employed by Stadie *et al.*(2). Freshly removed rat diaphragm was rinsed in cold phosphate buffer solution (.04 M sodium phosphate, .087 M sodium chloride, .005 M magnesium chloride, pH 6.8) to remove excess blood. Each hemi-diaphragm was used as such or divided into two parts. The tissues were weighed on Roller-Smith balance to the nearest mg and were then immersed (zero time) for 1 minute in phosphate buffer solution (.04 M, pH 6.8) containing unlabeled protein at various concentrations and tracer amount of I^{131} labeled protein. After 2 preliminary washings of 30 seconds each in 25 ml of phosphate buffer and 1 washing in 50 ml phosphate buffer, the tissue was assayed for radioactivity and was then placed in 50 ml oxygenated buffer solution at room temperature. Subsequent assays for radioactivity were made at intervals thereafter. For each assay the tissue was transferred to clean test tube containing 5 ml buffer solution and counted in the well scintillation counter. After each assay the tissue was again placed in 50 ml buffer solution through which oxygen was bubbled continuously. In 2 experiments the diaphragm was dried in air for 1 hour prior to incubation with insulin-I^{131}, and in 2 experiments diaphragm was immersed in 15% formaldehyde for 20 minutes prior to incubation with insulin-I^{131}. In several experiments a small piece of soft glass replaced the diaphragm but the procedure was otherwise identical to that outlined above.

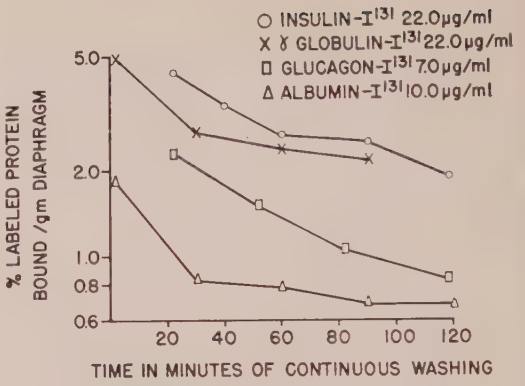

FIG. 1. Binding of various I^{131} labeled proteins to diaphragm and elution on continuous washing.

Results. With all I^{131} labeled proteins, radioactivity "bound" to diaphragm decreased significantly for about 1-1½ hours (Fig. 1). After this time a relative "plateau" was reached in which rate of loss of radioactivity was variable and did not exceed about 50%/hour. In most cases, however, progressive diminution in bound radioactivity occurred as long as observations were continued. In experiments described subsequently, the amount present at 1½ hours is taken as the amount "bound" unless otherwise indicated.

When concentration of insulin in the initial bathing solution was varied, the fraction bound to diaphragm showed no consistent correla-

* We are indebted to Drs. O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for generous supply of crystalline insulin.

† We are indebted to Drs. O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for generous supply of crystalline glucagon lot No. 208-15B-292A.

TABLE I. Influence of Insulin Concentration in Medium on Binding of Insulin by Diaphragm *In Vitro*.

Insulin conc. in medium, μg/ml	% bound per g tissue	μg insulin bound per g tissue
.09	1.6	.007
.30	7.7	.12
.41	15.6	.32
1.6	7.7	.61
2.0	2.24	.22
4.0	1.40	.28
5.6	7.80	2.20
7.0	1.70	.59
8.1	2.90	1.20
13.0	2.34	1.50
15.0	.51	.41
21.6	2.34	2.50
30.0	4.30	6.40
30.0	.26	.47
41.6	3.30	6.90
70.0	1.50	5.20
80.1	2.70	10.8
85.0	.21	1.07
101.6	.90	4.60
142.	1.16	8.20
198.	3.50	34.6
282.	1.30	18.0
302.	2.28	34.4
335.	.38	7.6
422.	1.10	23.2
485.	1.10	26.5
502.	.46	11.6
700.	.81	28.4
800.	.74	29.6
802.	.44	18.0
1005.	1.98	100.
1400.	.92	64.
2004.	.51	51.

tion with insulin concentrations in the range 0.09 - 500 μg/ml but, as a rule, was definitely decreased at higher concentrations (Table I). Consequently, there was no constant increase in absolute amount of insulin bound/g tissue with increase in concentration in the lower concentration range, nor was there a definite limiting value to the amount of bound insulin with increase in concentration up to limits of solubility of insulin at the pH employed (Table I). Such lack of consistency was observed not only among different experiments but frequently also when different pieces of the same diaphragm were tested simultaneously (Fig. 2). Variable adsorption to glassware with loss from bathing solutions was also observed at low insulin concentrations and was probably responsible for absence of reproducible adsorption isotherm; the data for binding as given, are expressed in terms of amounts still remaining in solution at beginning of incubation periods.

When the diaphragms were immersed in solutions containing tracer insulin-I^{131} and significant concentrations of unlabeled serum albumin or gamma globulin, radioactivity "bound" to diaphragm was strikingly diminished (Fig. 3). Similarly the binding of tracer albumin-I^{131} or γ globulin-I^{131} to diaphragm was inhibited by presence of high concentrations of heterologous unlabeled proteins (Table II). Of the 3 proteins, insulin appeared to compete most effectively for the binding of all.

Insulin binding, by air-dried and formaldehyde-treated diaphragms, was no less marked than by fresh diaphragm (Table III).

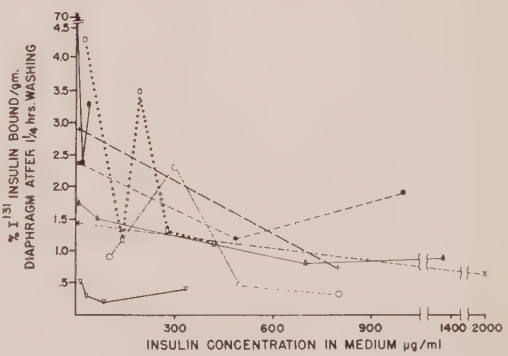

FIG. 2. Binding of I^{131} labeled insulin to diaphragm as function of insulin concentration. For each curve, experiments at different concentrations were run simultaneously with pieces of the same diaphragm.

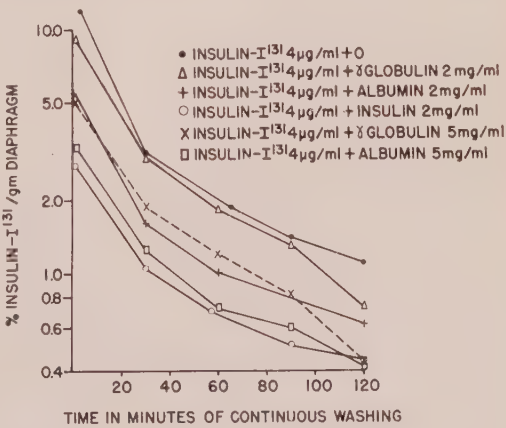

FIG. 3. Effect of high concentrations of homologous and heterologous proteins on binding of I^{131} labeled insulin to diaphragm.

TABLE II. Influence of Presence of Homologous and Heterologous Carrier Proteins on Binding of I^{131} Labeled Proteins by Diaphragm *In Vitro*.

Labeled protein	μg/ml	Carrier protein	μg/ml	% labeled protein bound per g diaphragm
Insulin-I^{131}	9.	0	0	1.70
	9.	I*	1,400	1.06
	4.	0	0	1.40
	4.	I	2,000	.51
	4.	A	2,000	.81
	4.	A	5,000	.60
	4.	γ-g	2,000	1.35
	4.	γ-g	5,000	.80
Albumin-I^{131}	6.	0	0	.58
	6.	I	800	.36
	20.	0	0	.67
	20.	I	2,000	.25
	10.	0	0	.65
	10.	A	500	.76
	10.	A	10,000	.33
	10.	γ-g	650	.65
	20.	γ-g	10,000	.25
γ-glob.-I^{131}	112.	0	0	2.16
	112.	I	2,000	1.01

* I = Insulin; A = Albumin; γ-g = γ-globulin.

TABLE III. Binding of Insulin *In Vitro* by Fresh Diaphragms and by Diaphragms Dried in Air or Treated with Formaldehyde.

Insulin conc. in medium, μg/ml	Condition of diaphragm		% insulin in medium bound per g tissue after washing 1 hr	1½ hr
6	Fresh	a	1.40	1.10
	"	b	1.50	1.28
6	Air-dried, 1 hr	a	2.40	1.80
	Idem	b	2.30	"
4	Fresh	a	2.46	1.85
	"	b	2.10	1.63
4	15% formaldehyde-treated, 20 min.	a	2.08	1.58
		b	"	"

When pieces of glass instead of rat hemidiaphragm were used, results on binding of insulin after one hour washing were quite similar to those in experiments with diaphragm, including evidence for a stronger competitive inhibitory effect of insulin than of γ globulin on binding of insulin-I^{131} (Table IV). However, the degree of inhibition by high concentrations of protein was much greater than in experiments with diaphragm even when the % adsorbed at trace levels was in the same range. In addition, rate of elution of both proteins from glass was significantly slower than from diaphragm.

Discussion. Adsorption of 4 different I^{131} labeled proteins to diaphragm has been demonstrated. Evidence for nonspecificity of this adsorption is presented in experiments demonstrating inhibition by heterologous proteins and by a similar pattern of adsorption observed with formaldehyde-treated diaphragms and diaphragms permitted to deteriorate in air. In support are the observations indicating adsorption and competitive inhibition on a biologically inert material, glass. Serum albumin and γ globulin are effective competitive inhibitors for the "binding" of insulin-I^{131} to isolated rat diaphragm and insulin is likewise an effective competitive inhibitor for the binding of either albumin-I^{131} or γ globulin-I^{131}.

It has previously been observed that insulin-I^{131}(4,5) and glucagon-I^{131}(7) adsorb quite firmly to paper, and that such adsorption is not significantly inhibited by the presence of moderate amounts of serum proteins but is inhibited competitively by the presence of high concentrations of homologous proteins. Depending on the nature of the binding sites and the reactive groups of the specific protein, a greater or lesser degree of selection may be observed with different adsorbing systems. In the case of diaphragm, paper and glass, insulin appears to be adsorbed preferentially in the presence of several other proteins but the diaphragm is not more selective than the inert materials.

Furthermore, several of our observations on insulin binding by fresh diaphragm are in dis-

TABLE IV. Binding of I^{131} Labeled Proteins by Glass in Presence and Absence of High Concentration of Homologous and Heterologous Proteins.

Labeled protein	μg ml	Carrier protein	μg/ml	% I^{131} protein per g glass
γ-glob.-I^{131}	1.6	0	0	1.8
"	"	I*	1,600	.25
Insulin-I^{131}	1.0	0	0	4.2
"	"	I	1,600	.10
"	"	0	0	5.8
"	"	γ-g	10,000	.54

* I = Insulin; γ-g = γ-globulin.

agreement with those of Stadie and coworkers (1,2). We have been unable to demonstrate either the permanent fixation of bound insulin-I^{131} or a limiting value for bound insulin-I^{131} within the range of solubility of insulin.

It is not the intent of the present report to deny that the biologic effects of insulin are exerted through combination with some cell-fixed enzyme system or substrate compound. It is generally accepted that a combination of some sort is necessary for a chemical reaction between substances to take place. However, the present studies do not support the concept that the binding of insulin-I^{131}, observed *in vitro* with isolated rat diaphragm is necessarily of any physiological significance, or that these observations reflect, in any way, a biologic process which takes place *in vivo*.

If a brief exposure of diaphragm to insulin suffices to augment glycogen accumulation on subsequent incubation with glucose(1), the results of the present study would then suggest that survival time of the altered state induced by insulin must persist appreciably beyond the time of reaction with insulin. An evaluation of this survival time would appear to be of interest.

Summary. Serum albumin, serum γ globulin, crystalline glucagon and crystalline insulin were observed to bind to diaphragm *in vitro* and to be eluted slowly on continuous washing with buffered solutions. A lack of reproducibility of binding of insulin at low concentrations is attributable in part to variable adsorption to glassware. A definite saturation of binding sites on diaphragm was not observed at the highest concentrations employed. Competitive inhibition was observed in the presence of heterologous proteins. Binding capacity was not altered by treatment of diaphragms with formaldehyde. Binding of insulin by isolated rat diaphragm *in vitro* is not demonstrably of biologic significance but is attributable to nonspecific surface adsorption of proteins.

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Susceptibility of Inbred Mice to Group A Streptococcal Infection.* (23076)

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Group A streptococci isolated directly from patients often are rich in M protein but avirulent to mice unless adapted by rapid successive transfer(1). This study was undertaken to compare susceptibility of mice of diverse genetic constitution to infection by representative group A streptococci. The objective of the investigation was to provide a sensitive experimental host-parasite system that could serve as a more adequate model for study of group A streptococcal infections of man and their late sequelae.

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Materials and methods. Inbred mice used differed significantly in genetic, anatomic, and physiologic constitution(2). BALB, C58,

and a substrain of DBA/2 mice from colonies maintained in our laboratory, and BALB/c, C57 Black (C57-Bl), and AKR mice from Jackson Memorial Laboratory, were 4 to 6 weeks old when used unless specified otherwise. Pen-bred Swiss albino mice, Tumblebrook strain, were included for comparative purposes. *Strains of group A streptococci* grown in Todd-Hewitt broth† at 37°C for 6 hours were diluted decimally in Todd-Hewitt broth maintained at 4°C. Organisms, 0.5 ml, inoculated intraperitoneally into test mice, observed 3 to 4 months, were quantitated by plate counts. The LD_{50} of organisms for mice, calculated by the method of Reed and Muench(4), was defined as number of organisms/0.5 ml of inoculum that killed 50% of test mice in 10 days. Not less than 12 mice were used for each dilution of test organism. Group A streptococci recovered from mice were typed to assure accurate identification.

Results. Susceptibility of inbred mice to group A streptococcal infection. Selection of a strain of *Streptococcus pyogenes,* type 18, to assess susceptibility of mice to infection was arbitrary. Table I reveals that test strains of inbred mice were extraordinarily susceptible to infection; commonly less than 10 organisms caused death. Because of limitations inherent in plate counts and LD_{50} calculations the precise minimal number of organisms required to kill mice was not analyzed. When these survey experiments were extended, it was observed that BALB female mice survived inocula lethal to males in 24 hours. *The possibility that sex or/and age influenced susceptibility of mice to infection* was explored by comparing susceptibility of BALB, Swiss, C58 and DBA/2 mice to infection by *S. pyogenes,* type 18. It is evident from Table II that: a) adult BALB females were significantly more resistant to infection than males, b) both male and female DBA/2 and C58 mice were highly susceptible to infection and c) both age and sex influenced resistance of Swiss mice to infection. *BALB male mice were used to assay pathogenicity of group A streptococci of diverse origin.* A total of 4 strains of *S. pyogenes,* type 18, were included in the test group. The results of this experiment (Table III) were conclusive: the pathogenicity of group A streptococci for inbred mice was not general but limited to a specific strain of a single serotype; organisms freshly isolated from clinical specimens were avirulent. *Host-parasite relationships resulting from infection of inbred mice by S. pyogenes,* type 18. Ideally, animals infected experimentally should provide a model that reflects accurately the host-parasite relationship in infections of man. An important feature of infection of BALB and C58 mice by *Streptococcus pyogenes,* type 18, was the spectrum of diseases produced. For example, male BALB mice, highly susceptible to infection,

TABLE I. Comparative Susceptibility of Inbred Mice to Infection by *Streptococcus pyogenes,* Type 18.

Strain of mice*	LD_{50}
Swiss	$10^{5.14}$
BALB	$<10^1$
BALB/c	"
C58	"
C57 Black	"
AKR	"
DBA/2	"

* Six-mo-old Swiss mice of both sexes, and 5- to 6-wk-old male inbred mice were used in this exp. The LD_{50} was defined as No. of organisms/0.5 ml of inoculum given intraper. that killed 50% of test mice in 10 days.

† Experience demonstrated that Todd-Hewitt broth affected results markedly; *freshly* made Todd-Hewitt broth prepared as described by Massell(3) gave consistent results. A study of biochemical components of Todd-Hewitt broth that limit infectivity of group A streptococci for mice is in progress.

TABLE II. Influence of Sex and Age on Susceptibility of Mice to Infection by *Streptococcus pyogenes,* Type 18.

Strain of mice	Sex of mice	Age of mice (mo)	LD_{50}
BALB	♂	6	$<10^1$
	♀	6	$10^{3.8}$
	♂ or ♀	1	$<10^1$
Swiss	♂	3	$10^{4.9}$
	♀	3	$10^{6.1}$
	♂ and ♀	6	$10^{5.14}$
	" " "	1	$10^{2.56}$
C58	♂	6	$<10^1$
	♀	1	$10^{1.5}$
DBA/2	♂ or ♀	1	$<10^1$

TABLE III. Infectivity of Group A Streptococci of Diverse Origin for Inbred Male BALB Mice.

Strain of streptococcus	Origin of organism	LD_{50}
Type 1	Freshly isolated from clinical streptococcal septicemia	$10^{5.78}$
1-L	Mouse adapted strain of type 1 organisms*	$10^{2.12}$
4-Ase	Non-encapsulated strain of *Streptococcus pyogenes* known to produce large quantities of hyaluronidase	$>10^{7.0}$
12	Stock laboratory strain	"
18 M	Isolated from abscess of monkey; passed once in Todd-Hewitt broth	$10^{5.9}$
18 P	Substrain of Type 18 M isolated from laboratory worker infected accidentally; passed once in Todd-Hewitt broth	$10^{1.84}$
18	Substrain of Type 18 P passed once through skin of rabbit as described by Watson(5)	$<10^{1}$
18-4397B	Stock laboratory strain	$10^{5.57}$
19-1236	Freshly isolated from patient with streptococcal conjunctivitis; passed once in Todd-Hewitt broth	$>10^{7}$
28	Stock laboratory strain	"

* Kindly sent by Dr. R. Lancefield.

died without remarkable gross pathologic changes. In contrast, adult BALB female mice that survived an LD_{50} dose of organisms provided a consistent pattern of reaction to infection: suppurative lesions formed at the site of inoculation or in the extremities, became necrotic and sloughed. In these mice chronic infection persisted for 1 to 4 months to eventuate in death in most cases. In a small number of cases (1 to 2%) C58 mice that survived infection remained asymptomatic until the third month after inoculation when suppurative lesions occurred in joints and limbs. Organisms recovered from BALB and C58 mice were identified by cultural and serologic tests as *Streptococcus pyogenes,* type 18. Thus, survival of group A streptococci in mice for 3 to 4 months was established. Swiss mice that survived infection, in distinction to BALB and C58 mice, were not ill at any time nor was there suggestive evidence of chronic infection.

Discussion. An analysis of host-parasite relationships is facilitated by the availability of a sensitive assay system for factors which govern the virulence of an organism or determine resistance or susceptibility of the host. The finding(6) that inbred mice were highly susceptible to infection by an unadapted group A streptococcus could be significant since it may contribute toward elucidation of the complex factors involved in the reaction of the host to streptococcal infection. An indication of the nature of these factors was suggested by observation that: a) only a single strain of serotypes of group A streptococci tested was highly virulent to mice; b) sex and age of mice influenced susceptibility to infection; c) the genetic constitution of mice markedly controlled reaction to virulent organisms; and d) latent streptococcal infection persisted for 3 to 4 months in C58 mice. Denny and Thomas(7) reported survival for 104 days of group A streptococci in tissues of rabbits infected experimentally. The finding that group A streptococci could survive for relatively long periods of time in mice lends credence to the view that latent infection may be important in the induction of late sequelae to streptococcal infection.

Summary. Less than 10 organisms of an unadapted strain of *Streptococcus pyogenes,* type 18, caused fulminant infection in BALB, BALB/c, AKR, DBA/2, C58 and C57 Black inbred mice. Pen bred Swiss albino mice were significantly more resistant to infection. Inbred BALB and C58 mice that survived the original inoculum underwent latent or chronic infection; organisms were recoverable from untreated mice for at least 3 to 4 months. Data were obtained which revealed that susceptibility of mice to infection depended on strain of serotype of the organism tested as well as the genetic constitution, age and sex of the host. These studies demonstrated that a spectrum of diseases could be produced by employing a single strain of *Streptococcus pyogenes,* type 18, and different strains of inbred mice. The application of these findings to study of strep-

tococcal infections and their late sequelae was discussed.

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Developing Blood Brain Barrier to Trypan Blue.* (23077)

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Ever since Ehrlich first observed that the central nervous system could not be stained by intravenous injections of acid aniline dyes (1), information dealing with the functional and structural embryonic development of the blood brain barrier has been of considerable importance to physiologists. Bakay(2) was able to show by use of radioactive P^{32} that embryonic rabbit brain was more permeable to this ion than adult brain, and that permeability to P^{32} is greatest in early intrauterine life but continues to decrease until about 7 weeks of postnatal development. Grontoft (3) perfused human and animal fetuses with trypan blue and found the brain normally impermeable to this vital dye. Ten-minute delivered human fetuses 5 to 30 cm long had already developed an impermeability to trypan blue. It appears, however, that no one has observed penetration of vital dyes into the embryonic nervous system *in vivo*, due to the permeability problem of placental barrier and the difficulty of injecting embryos directly, although Waddington and Carter(4) were able to induce abnormalities in mouse embryos by injection of trypan blue into pregnant females.

In this study, an attempt was made to show *in vivo* staining characteristics of the rat embryo with trypan blue, and special emphasis was placed on development of blood brain barrier with respect to developing CNS blood vessels. All observations are made *in vivo* with the exception of the youngest rat embryos (10½ days, about 3 mm), which were perfused with trypan blue in Ringer's Solution while the heart was still beating.

Methods. Four-month-old Wistar female rats were used, and exact gestation times were determined by vaginal smears. If insemination had occurred, the female was separated from the male and used for experimentation at the appropriate gestation time. An error of about 12 hours in embryo age is possible with this method. Rat embryos were injected intraperitoneally or via amniotic circulation with a saturated trypan blue solution. Gestation ages in litters studied ranged from 10½ days to birth. Embryos of 10½ and 11 days were perfused with dye after delivery of the fetuses into Ringer's Solution at 37°C since it was extremely difficult to make successful *in vivo* injections in embryos younger than 12 days. Following injection, these fetuses were placed immediately in 10% formalin. All *in vivo* injections were made through the wall of the uterus, which was rendered transparent with proper illumination. Intravascular injections of the amniotic circulation were suc-

* Assisted by grant from United Cerebral Palsy Fn.

cessful in embryos of 13 days or older; but in 12-day-old fetuses, intraperitoneal injections of dye were administered. Mico-capillary pyrex pipettes were used for all injections. The pregnant females were prepared under ether anesthesia, and the uterine horns were exposed. Injections were controlled with oral pressure by means of a small rubber-blood pipete tube and were visualized under a dissecting microscope. This method was found most advantageous since it allowed greatest manipulation of the pregnant uterus. Six to 24 hours after *in vivo* injections, the embryos were delivered, and both injected and control embryos were fixed in 10% formalin. Only injected embryos that were delivered alive and living control embryos of the same litter were used for observation. Certain embryos were injected directly into the ventricular system of the developing brain to demonstrate the staining of the nervous system by dye not confined to blood vessels.

Results. Intrauterine injections of trypan blue were performed intraperitoneally or intravascularly in 100 rat embryos ranging from 10 days to birth. Usually 2 fetuses in a litter were injected. Out of the 100 embryos injected, 60 were successful; the remaining 40 died *in utero* and were not used in this study. In 20-day embryos injected on nineteenth day of gestation, there was no evidence of trypan blue in the central nervous system following a 24-hour dye equilibrium period, although all other organs showed intense staining with the vital dye. When trypan blue was injected into the ventricular system of developing brain, there was complete staining not only of the central nervous system but also of the rest of the embryo.

Trypan-blue injected rat embryos of 17, 15, and 12 days gave essentially the same results (Fig. 1). In each case, trypan blue had been administered intraperitoneally 6 hours prior to delivery of embryos. Although skin, viscera and cerebral vessels showed intense staining, there was no evidence of dye penetration within the central nervous system. In 12- and 10-day embryos the barrier phenomenon appears most distinctly in the developing spinal cord; however, it definitely existed in brain, even though in the earlier embryos there was still incomplete closure of the neural folds.

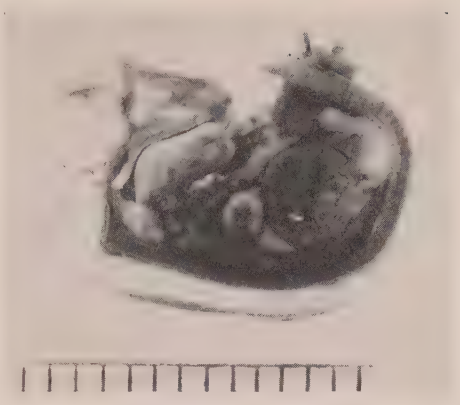

FIG. 1. Fifteen-day-old rat embryo injected with trypan blue via amniotic circulation and sacrificed 6 hr after injection. There is no evidence of dye in the central nervous system although dye penetrated the rest of the body. Scale in mm.

Discussion. The existence of a blood brain barrier phenomenon to trypan blue was demonstrated in this study in rat embryos ranging in age from the time blood vessels invade the brain until birth. Vascular invasion of the central nervous system occurs in the 3 to 4.5 mm rat embryo, which is approximately at 10 days of gestation(5). Our observations are in agreement with those of Grontoft(3), who perfused a great number of animal and human fetuses with trypan blue. When he perfused fetuses within 45 minutes after separation from the mother, the central nervous system remained impermeable to trypan blue. It was his belief that as long as the endothelium of CNS capillaries was intact, the central nervous system was impermeable to trypan blue.

Penfield(6) indicated that astrocytes make their appearance in the developing central nervous system at about the same time that blood vessels invade the brain. At birth, astrocytes are not well developed and have only a few fibrous processes, and it would appear that embryonic impermeability to trypan blue is unrelated to developing astrocytes but rather to vascular endothelium of the cerebral blood vessels. In fluorescent dye studies, the findings of Rodriguez(7) additionally support this hypothesis. It cannot be as-

sumed, however, that the mechanisms preventing all substances from entering the central nervous system are the same as those which retard protein-bound dyes.

Hess(8,9) believes that presence of ground substance in the central nervous system is necessary for the functional blood brain barrier. He observed that in neonatal mice and several other newborn animals the periodic acid Schiff reaction was negative, and he concluded that the absence of a chemical "ground substance" was causally related to the lack of blood brain barrier activity. We have observed that a blood brain barrier toward trypan blue definitely exists in neonatal rats. Other unpublished observations lead us to believe that this is also true in neonatal mice and cats. Behnsen(10) observed that areas which normally stain with trypan blue in the adult mouse were somewhat larger in the neonatal animal, but his findings have been misinterpreted by many investigators as pointing to an absence of blood brain barrier activity *in utero*.

As Bakay(2) points out, it seems that the blood brain barrier is not simply a screening agent but a complex mechanism which is functionally adapted to the needs of the central nervous system. The phenomenon toward trypan blue in the rat exists as soon as the vessels are laid down in the developing brain, but the same mechanism may not act for all substances. The selectivity for smaller molecular weight substances such as the electrolytes is decreased as the embryo grows older and might be correlated with neuroglial development.

Summary. Trypan blue injected into developing rat embryos (10 days to birth) fails to stain the central nervous system. This impermeability in the developing central nervous system exists at the time when blood vessels invade the brain. It cannot be assumed, however, that the mechanisms which prevent protein-bound dyes from penetrating the cerebral blood vessels are the same for all other substances.

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Pharmacological and Biochemical Study of a Carcinoid Tumor.* (23078)

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The high concentration of serotonin (5-hydroxytryptamine, 5-HT) in malignant carcinoids accounts, at least in part, for the striking symptoms often experienced by patients with this disease(1,2,3). Serotonin is formed by ring-oxidation and subsequent decarboxylation of tryptophane, a pathway that consumes as much as 60% of dietary tryptophane in carcinoid patients compared with 1% in normal individuals(4,5). Another pharmacologically active compound, chromatographically distinct from serotonin, but as yet unidentified, has been obtained from malignant carcinoids(6,7,8). In this work, we have measured both the serotonin content and the activity of 5-hydroxytryptophane decarboxylase in a benign appendiceal carcinoid, and at the same time searched for other pharmacologically active compounds in this tumor.

Methods. The appendix was removed routinely during a pelvic laparotomy on a 48-year-old white housewife. At tip of the appendix, the mucosa and sub-mucosa were replaced by firm, grayish yellow tissue which was identified microscopically as an argentaffin-staining carcinoid tumor. The fibromuscular coat of the appendix was dissected free from the carcinoid. The tan mucosa of a grossly and microscopically normal appendix was dissected from the underlying muscular coat and used as control material. 5-hydroxytryptophane decarboxylase activity in a portion (150 mg) of both appendiceal tissues was estimated by a method reported earlier (9). The remainder of the carcinoid (210 mg) was extracted with 95% acetone and petroleum ether and taken to dryness *in vacuo* (10). Both the extract and residue were kept at −15°C until analyzed. Similarly, blood drawn from the antecubital vein of the patient after removal of the appendix was extracted with acetone and dried. The residue remaining after acetone extraction of the carcinoid was extracted with 0.2 *N* HCl(7) and taken to dryness. Hereafter in the text, this is referred to as the "acid extract." Bioassays for serotonin were carried out on the heart of *Venus mercenaria*,‡ on the rat's uterus, in estrus, and on the ileum of the guinea pig. Each biological indicator was suspended in a 4 ml overflow bath with a constant flow of air. The heart of *Venus mercenaria*(11) was bathed in a nutrient medium previously described(12), to which was added benzoquinonium chloride (6 mg/l) to block the inhibitory effect of esters of choline. Uteri from rats treated with stilbestrol (100 μg/100 g) on the preceding day were placed in de Jalon's solution with atropine sulfate (1 mg/l). Substance P, kindly presented by Dr. T. B. B. Crawford of Edinburgh, was used as a reference substance in tests on the ileum, suspended in Tyrode's solution containing 0.1 mg of atropine sulfate and 1 mg of mepyramine maleate per liter. Ascending paper chromatography in isopropanol and 0.1 *N* HCl (7:3), was carried out on acetone soluble extracts in 8% aqueous NaCl, and in the upper layer of a system containing *n*-butanol, glacial acetic acid, and water (4:1:5). A solution of the acetone insoluble material in 0.1 *N* HCl was chromatographed by using the first mentioned solvent mixture. After drying, the papers were cut into pieces, each corresponding to 1/30th of the whole migration length, eluted with NaCl, and the eluates were tested on Venus hearts. In addition, guinea pig ileum was employed for testing all isopropanol papers, and in the case of the acetone-insoluble part, rat uterus also

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TABLE I. Serotonin-Forming Activity of Carcinoid and "Normal" Tissue.

Tissue	5-Hydroxytryptophane decarboxylase activity* (μg 5-HT formed per g per hr)	Serotonin in fresh tissue (μg per g)
Appendiceal carcinoid	840	952
Normal appendiceal tissue	23	1

* In terms of serotonin formed from 4×10^{-4} *M* 5-hydroxytryptophane under the standard conditions established by Gaddum and Giarman(9).

was used. Not only were the paper strips examined for fluorescent material(13) but also the extracts were examined for their fluorescence characteristics in an Aminco-Bowman Spectrophotofluorometer. The extracts and standard materials were examined routinely in a 0.1 *N* HCl-medium. In some cases the fluorescence spectrum in 0.1 *N* HCl was compared with that in a 3 *N* HCl, because activation of serotonin, at 295 mμ in dilute acid results in a peak fluorescence emission at 330 mμ, while this shifts to 550 mμ in 3 *N* HCl (14).

Results. Table I shows the comparison between natural serotonin content and serotonin-forming capacity of each tissue. Thus, it is clear that the carcinoid contained nearly 1000 times as much serotonin as normal appendiceal tissue, and had about 35 times the capacity of the normal appendix to form serotonin.

After surgery the blood of the patient contained 3.7 μg of serotonin per 100 ml, a value within the wide range of normal values obtained in this laboratory(15). A 24-hour sample of urine, obtained 2 days post-operatively, gave a negative test for carcinoid, as determined by the urinary level of 5-hydroxyindole acetic acid(4).

Lysergic acid diethylamide (LSD-25), which specifically inhibits the stimulatory effect of serotonoin on the uterus of the rat(16) completely blocked the effect of the acetone extract of the carcinoid on this preparation. On both the clam heart and the guinea pig ileum there was only one spot of activity on paper strips developed with the 3 solvent systems and this spot corresponded to the R_f values of a sample of serotonin, chromatographed concomitantly, either separately or mixed with the extract. Bufotenine (dimethylserotonin hydrochloride) migrated faster in all solvent systems and differed qualitatively from serotonin and the extract in its action on the clam heart and showed a persistent effect in contrast with the evanescent effects of serotonin and the extract. Only the pharmacologically active area of the paper on which the extract was chromatographed showed fluorescence. Similarly, the acid extract of the carcinoid showed activity on the heart, uterus, and ileum, and the R_f values of the active spots corresponded to the R_f values of serotonin. The acid extract showed no bufotenine-like activity on the clam heart. LSD-25 prevented response of the uterus to the acid extract.

Spectrophotofluorometric studies indicated that serotonin was present in both the acetone and acid extracts of the carcinoid and of the normal appendix. Other fluorescent materials, probably not indoles, were found in the acid extracts. Table II summarizes these data.

Discussion. It is now a well established fact that carcinoid tumors contain relatively massive concentrations of serotonin(6,7,8). We have obtained data to substantiate this and have shown further, by direct estimation, that the increase in serotonin parallels an augmented capacity of the tissue to decarboxylate 5-hydroxytryptophane. This agrees with the previous finding that carcinoid tissue was

TABLE II. Spectrophotofluorometric Characteristics of Extracts of a Carcinoid Tumor.

	Peak fluorescence (mμ)			
	0.1 *N* HCl			3 *N* HCl
Activation wavelength (mμ)	295	310	325	295
Substance studied				
Serotonin	330			550
Carcinoid				
Acetone extract	330			550
Acid extract*	330	630	670	550
Normal appendix				
Acetone extract	330			550
Acid extract*	330	650		550

* Of acetone insoluble portion.

more active than kidney cortex and liver in decarboxylating 5-hydroxytryptophane (17). That the blood levels of serotonin and the urinary levels of 5-hydroxyindole acetic acid were in the normal range post-operatively in the case described indicates that the tumor removed was the primary one and that metastases retaining the capacity to form serotonin are not likely to have been present.

Of special interest have been the reports by Lembeck that carcinoids contained, in addition to serotonin, a second pharmacologically active substance, which was not identical with substance P(6,7,8). However, study failed to reveal a second substance, and all the pharmacological effects and fluorescence characteristics of our extracts could be attributed to their content of serotonin. It is important to point out in this connection that not all the carcinoids studied by Lembeck were found to contain the second active substance. It would seem worthwhile to continue to seek such a substance in other carcinoids.

Summary. The pharmacological activity and fluorescence characteristics of extracts of an appendiceal carcinoid tumor could be accounted for entirely by its serotonin content. The amount of serotonin in the carcinoid tissue was about 1000 times that of normal tissue, and its enzymatic ability to form serotonin from 5-hydroxytryptophane was about 35 times that of normal human appendiceal tissue.

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Effects of Induced States on Tissue Lysozyme Activity.*†‡ (23079)

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To establish a model system for studies of protein biosynthesis as it may be affected by hormonal conditions, experiments to test possible alteration in activity of tissue lysozymes in various hormonal states were undertaken. Although it has been observed that ACTH therapy produced a marked fall in fecal lysozyme coinciding with clinical improvement of ulcerative colitis(1) and that intravenous administration of insulin also caused a decrease in total output of the enzyme in gastric juice(2), no reports have been seen to indicate the possible role of hormones in controlling *in vivo* levels of tissue anti-bacterial enzymes.

Methods. The experimental animals, obtained from the Charles River Breeding Laboratories and of the Sprague-Dawley strain were males, weighing between 50-75 g. Hypophysectomy was carried out at 50 days of age and the animals were received at 52 days of age at which time each rat was injected with 5000 units of penicillin G (Squibb). Hypophysectomy was confirmed by adrenal weights at term and examination of the *sella turcica*. Animals were housed individually in screen-bottom cages and received diet and water *ad libitum*. Diet consisted of the following: lactalbumin (Labco, Borden Co.) 30%; vitamin mix(3) 2%; sucrose, 58%; Salts IV(4), 4%; corn oil, 5%; and fat soluble vitamins, 1% (Nopco Chemical Co. A and D concentrate and cod liver oil; 300 I.U. vit. D/g, 2250 U.S.P. units vit. A/g). Food consumption was determined on alternate days by weight loss in food cups where little or no spillage occurred. Beef GH (Armour), lot M-108 (activity = 75% of Armour standard + 0.15 U.S.P. unit/g of thyrotropic hormone contaminant) was administered as follows: 0.2 mg dissolved in 0.1 ml 0.85% NaCl was injected daily by intraperitoneal route. ACTH (Upjohn) was dissolved in physiological saline and 1 U.S.P. unit in 0.1 ml solution was injected intraperitoneally each day. Thyroid concentrate (E. Lilly; lot Z-3096; 0.17-0.23% iodine) was administered orally as 1% of diet. Adrenal weights were taken after the fresh glands were teased free of surrounding tissue and blotted. The following tissues were excised, individually wrapped in foil and placed in freezer: liver, lung, spleen, kidney and intestine (from pyloric valve to the cecum). Previous washing of the intestinal lumen caused a loss of about 5% of total enzyme activity of this portion, therefore no washing was attempted. The enzyme activity in 30 cm segments of the tract, caudad from the pyloric valve, demonstrated a different pattern in the growing rat compared to the adult rat; therefore the entire segment from pylorus to cecum was homogenized for the enzyme assay. Lysozyme activity was determined by previously described photometric method(5) using *Micrococcus lysodeikticus* as substrate prepared according to Litwack and Pramer(6). Tissue nitrogen was determined with the Folin-Ciocalteu reagent essentially as described by Lowry, *et al.* (7) and Oyama and Eagle(8). A nitrogen value is obtained by converting the Folin color which has been standardized with a tissue preparation whose Kjeldahl nitrogen value had also been determined. Linearity between the Folin color and Kjeldahl nitrogen content was obtained. Optical density values were read at 660 mμ to 0.4. Lysozyme specific activity is defined as μg equivalent of crystalline

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† The following abbreviations will be used: GH (growth hormone); ACTH (adrenocorticotropic hormone); HYPOX (Hypophysectomy or hypophysectomized).

‡ The author is indebted to Dr. S. L. Steelman of Armour and Co. for a gift of beef GH. The technical assistance of Miss Sarah W. Perry in some of the enzyme assays is gratefully acknowledged.

TABLE I. Effects of Induced States on Tissue Lysozyme Activity.*

Group	No. animals	Lysozyme specific activity (μg EEWL†/mg N)				
		Liver	Lung	Intestine	Kidney	Spleen
Normal control	11	5.3 ± 1.7‡	98 ± 9	14.8 ± 3	87 ± 15	37.2 ± 4
HYPOX	11	12.0 ± 5.3	122 ± 13	15.2 ± 9	166 ± 17	34.6 ± 5
" + ACTH	12	5.3 ± 1.7	108 ± 5	15.2 ± 4	141 ± 17	39.8 ± 3
" + GH	9	9.3 ± 1.7	96 ± 7	15.2 ± 4	119 ± 5	38.6 ± 3

* Animals on exp. 25 to 28 days. † EEWL = equivalent to crystalline egg white lysozyme.

‡ Stand. error of mean $= \frac{\sqrt{\Sigma d^2/n-1}}{\sqrt{n}}$.

egg white lysozyme (Armour) taken from a standard curve(5)/mg tissue Folin nitrogen (μg EEWL/mg N). At the time of analysis, the tissues were thawed§ for 30 min. at 5°C, teased free of surrounding tissues, blotted and weighed. Homogenates were made by grinding in 5 volumes of cold distilled water added to the tissue. These were diluted 10 times for enzyme and nitrogen determinations at 25°C. Each extract was assayed with an accuracy of ± 0.2% transmission/30 sec.(5). This accuracy was always duplicated within 2 to 3 determinations. All enzyme activities were made using the same sample of dried *M. lysodeikticus* cells. (Δ% transmission/30 sec. of 1.6 is equivalent to 5 μg of Armour egg white lysozyme.) Experiments were carried out in 2 durations, the first for 25 to 28 days and the second from 8 to 12 days.

Results. Growth data under various hormonal conditions were in agreement with those reported by Chow and Greep(9) who used a similar diet. Table I shows the results of a long-term experiment in which tissue lysozyme activity is reported as specific activity (SA). Measurements of SA show a remarkable consistency in the spleen regardless of the hormonal condition of the animal. Average body weights of animals at completion of the experiment were as follows: Normal, 248 ± 5 g (standard error of mean); HYPOX, 72 ± 4 g; HYPOX + ACTH, 80 ± 3 g and HYPOX + GH, 130 ± 4 g. The liver, lung and intestine also remain constant in the various groups. The most striking changes of the tissues tested occur in the kidney where the enzyme is most heavily concentrated(5). Upon HYPOX the SA of the enzyme is doubled even though the animals' body weight has failed to increase by about 75%. ACTH does not significantly alter this change, but GH has a slight depressing effect upon the enzyme SA. In the case of the kidney enzyme, a marked relationship of control through "capping" the enzyme activity via pituitary secretion appears to exist. The depressing effect of GH may be significant but since depression is not accomplished to the level of the untreated control, it is likely that other factors play a role in controlling the kidney enzyme SA.

A second short term experiment is shown in Table II. A study of the effects of thyroid hormone was necessary to establish if the thyrotropic hormone contaminant of GH was playing a part in the observed depression of enzyme SA. This experiment confirms the marked rise in kidney enzyme SA after 8 days of HYPOX. Levels of SA of lysozyme in liver, lung, intestine and spleen were constant in untreated and HYPOX animals. The effects of the thyroid hormone are great, especially upon the liver, lung, intestinal and kidney enzymes. When the unoperated animals received thyroid for 8 days, SA of the enzyme of all tissues except the spleen were reduced up to 50%. When the HYPOX group was treated with thyroid, a similar reduction in SA followed. The superimposed thyroid stress upon HYPOX reduced SA of the kidney enzyme to a value slightly below that observed in untreated control. When the changes in body weight are considered these differences appear to be greater. Average body weight of each group at termination of the experiment was: Normal, 157 g; HY-

§ No loss in activity was experienced before and after freezing regardless of storage time.

TABLE II. Effects of Hypophysectomy and Thyroid Hormone on Tissue Lysozyme Activity.*

Group	No. animals	Lysozyme specific activity (μg EEWL†/mg N)				
		Liver	Lung	Intestine	Kidney	Spleen
Normal control	4	5.3 (1–12)‡	141 (81–190)	32 (11–66)	108 (92–131)	47 (39–53)
Idem + 1% thyroid	3	2.7 (2.6–4)	98 (85–105)	16 (11–21)	59 (48– 66)	40 (36–45)
HYPOX	5	9.3 (4–18)	137 (74–192)	20 (5–36)	155 (108–190)	47 (25–68)
" + 1% thyroid	7	3.8 (1–11)	109 (91–126)	6.7(3–29)	87 (65–120)	43 (24–61)

* Animals on exp. 8-12 days. † EEWL = equivalent to crystalline egg white lysozyme. ‡ Range.

POX, 82 g; HYPOX + thyroid, 67 g; and normal + thyroid, 118 g. Decreased weights due to thyroid stress were accompanied by decreased food intake. It appears, however, that this factor was not important in reduction of tissue enzyme SA because the liver xanthine oxidase activity(10,11) of these animals was tested and was not reduced when thyroid was given to normal animals.

Discussion. Increased SA of kidney lysozyme after hypophysectomy suggests that the anterior pituitary may control the SA of the kidney enzyme. It seems likely that thyrotropic hormone would play a large indirect role. The further slight suppressive effect of GH suggests that GH may play a part in this suppression. ACTH does not alter lysozyme activity in uninfected tissues, as found in ulcerative colitis(1). The "capping" effect of an acidic short chain peptide which could enter a kidney cell and inactivate the cytoplasmic lysozyme is an attractive hypothesis to explain the hypophysectomized effect. Several questions are raised as a result of these experiments, primarily, role of the kidney as a depository for the enzyme. Since the kidney appears to accumulate the greatest tissue activity of the enzyme, it may be that the kidney promotes a highly active synthesis of lysozyme. This possibility is being investigated. It is known, however, that lysozyme also circulates in blood(5,12). It is interesting to reflect that the data reported here demonstrate a remarkable consistency of the spleen SA. It might be suggested that the enzyme could be formed by the spleen and circulated to tissues which accumulate it, analogous, perhaps, to a hormone secretion where a concentration gradient is maintained between the blood stream and the concentrating tissues.

The effect of inhibition by thyroid hormone of kidney lysozyme SA and SA of other tissues occurs in untreated or HYPOX animals and is very pronounced. Fraenkel-Conrat (13) has demonstrated that lysozyme binds iodine which results in reversible inactivation. It was concluded that the imidazole group participated in the reaction. It seems possible that the thyroid hormone might bind lysozyme although why the kidney would appear as the main target tissue remains a problem. It has been shown that the action of some factors, such as insulin, in decreasing the titer of lysozyme, do so by binding the enzyme and in some cases precipitating it (14). If it may be assumed that kidney lysozyme is also in the bound form, the measurable activity of the tissue is due to the free form of the enzyme which becomes dissociated because of effects of various factors, in this case hormonal effects, upon association of the enzyme. One could then view hypophysectomy as having a "dissociating" effect and thyroid hormone as having a depressing or "associating" effect. In this regard it is of interest that Smith and Dubos(15) have reported that thyroxine treatment "interfered with the bactericidal mechanism in the liver, spleen and kidneys of mice during the initial phase of infection." In addition, they found that thyroid extract or dinitrophenol were inactive in causing the infecting organisms (staphylococci) to multiply more rapidly in the various organs.

Summary. 1. In growing rats, hypophysectomy has been shown to significantly increase the level of kidney lysozyme specific activity(SA) up to 2-fold. Other tissues, particularly the spleen, show constant SA regardless of hormonal condition of the animal. 2. Thyroid has a marked depressing

effect upon kidney lysozyme SA when given to either untreated or hypophysectomized growing animals.

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Development and Serial Cell-Free Passage of a Highly Potent Strain of Mouse Leukemia Virus.* (23080)

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After it was found that filtered extracts prepared from tissues of leukemic Ak or C58 mice may induce leukemia following inoculation into newborn mice of a susceptible strain (1-4), it became apparent that the results of individual experiments may vary to a considerable degree, depending on the particular extract used. Of 70 filtrates tested, each prepared from a different donor, 18 were found completely inactive(5). Similar observations were made by Woolley(6). Furthermore, extracts prepared from C3H mice in which leukemia had been induced with cell-free Ak leukemic agent, proved more active than those prepared from Ak mice with spontaneous leukemia(4-5). It appeared reasonable to assume, therefore, that selecting a particularly active extract and passing the agent through several successive newborn hosts, may eventually result in a strain of leukemic virus of high potency(5,7). To find a potent extract, groups of litters of newborn C3H mice were inoculated with cell-free leukemic extracts prepared from different donors. Among inoculated mice, those developing leukemia at earliest date were then used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice. The first mice developing leukemia were used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice, etc. A number of different passage-strains of leukemic agents were thus obtained. Of the leukemic passage-strains developed during these experiments, the one here reported and designated "passage A" proved to be highly pathogenic. Filtered extracts prepared from the last few passages of this agent induced acute, generalized, lymphatic leukemia in most of the inoculated mice after only 3½ months. Furthermore, extracts containing the passage virus could be preserved by lyophilization, or simply by freezing in carbon dioxide ice at –70°C in sealed ampoules without apparent loss of activity.

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Methods. Preparation of leukemic extracts.

From mice with advanced leukemia, parts of liver, spleen, mediastinal and mesenteric tumors, and peripheral lymph nodes, were removed aseptically, weighed, ground in mortar with sterile physiological saline added to obtain 20% concentration, centrifuged at 0°C, first at 3000 rpm (1400 x g), for 15 minutes, then at 9500 rpm (7000 x g), for 5 to 10 minutes; the final supernate was used for inoculation. In experiments in which a filtrate was to be inoculated, approximately 10 ml of the 9,500 rpm supernate was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli,* passed through Selas, porosity 02 or 03, porcelain filter candle, and the filtrate then inoculated into newborn mice (in each instance the filter candle retained *E. coli*). In a few experiments the 9,500 rpm supernate, or the filtrate, were centrifuged in Spinco L ultracentrifuge at 40,000 rpm (144,000 x g) for a few minutes to 3 hours, to concentrate the virus; the resuspended sediment pellet was then used for inoculation. In one experiment (fourth passage), blood plasma of the leukemic donor was used for preparation of the extract. All extracts were kept at 0°C, and injected within 48 hours. *Test animals.* Newborn C3H, or foster nursed C3H(f) mice, both of Bittner substrain(4), were used for inoculations. Most animals were <12 hours, many <6 hours, none >16 hours old, at time of inoculation. Those mice that died when less than 2 months old were not included in the tabulation; this concerns particularly the mortality, usually, however, not exceeding 25%, occurring among infant mice within hours or days after inoculation.

Results. Serial, cell-free passage of the leukemic agent from host to host. Only a cell-free passage, consisting of successful transmission of the leukemic agent from host to host, inoculating either a centrifuged or filtered extract, was considered a consecutive "passage," and given a passage number. In one instance, when after the fifth passage newborn mice were not immediately available for inoculation, yet the donor had to be sacrificed, leukemia was carried for 3 successive transplantations by intraperitoneal cell-grafts into adult hosts of the same C3H inbred line; such cell-transfer, however, was considered to be within the same passage, and was designated by the same passage number, *i.e.* "passage 5-a," "5-b" and "5-c." Only succeeding cell-free transfer was then designated "6th passage." This procedure was adopted because it appeared questionable whether cell-transfer would increase, or even sustain, the infective potency of the leukemic agent. Transplantations were carried out at a time when sufficient information was not yet available to suggest that the leukemic agent could be preserved by freezing at −70°C.

Origin of the leukemic virus strain. A breeding female mouse No. 557 of the Ak-n strain of our colony, which developed spontaneously lymphatic leukemia at 7½ months of age, was sacrificed; liver, spleen, mesenteric and mediastinal tumors, removed aseptically, were ground, with physiological saline solution added to make a cell suspension of 20% concentration. This cell suspension was then centrifuged at 3,000 rpm for 15 minutes, and the supernate was dry-frozen.

First passage. The lyophilized extract was preserved in a sealed ampoule in refrigerator at 4°C for 5½ months. The content of the ampoule was then resuspended in 2 ml of sterile physiological saline, and inoculated into a newborn C3H litter consisting of 3 females and 4 males. All 3 females developed generalized leukemia at 4½ to 5½ months of age; of the 4 inoculated males, 3 developed leukemia at 4½, 5½ and 10½ months of age, and 1 died at 10½ months with no evidence of tumors or leukemia.

Second passage. One of the leukemic females from the preceding experiment served as donor for preparation of an extract. The leukemic cell suspension was centrifuged first at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. The second supernate was centrifuged in Spinco Model L Ultracentrifuge (rotor No. 40) at 40,000 rpm (144,000 x g) for 90 minutes. The sediment pellet from final centrifugation resuspended in 2 ml of sterile physiological saline, was inoculated into a newborn C3H(f) litter consisting of 2 females and 1 male. All 3 inoculated mice de-

veloped generalized leukemia after 1½ to 3½ months.

Third passage. One of the leukemic females from the preceding passage was used as donor. The leukemic cell suspension was first centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. The second supernate was then centrifuged in Spinco Ultracentrifuge for 3 hours at 40,000 rpm (144,000 x g); the sediment pellet from final centrifugation was resuspended in 2 ml of physiological saline solution and inoculated into 5 newborn C3H males: 2 developed leukemia at 6 and 7½ months of age respectively; of the remaining 3 mice, 1 died when 4 months old and 2 at 17 months without signs of either tumors or leukemia.

Fourth passage. From a leukemic male from the preceding passage, in ether anesthesia, 0.5 ml of blood was removed directly from heart and immediately diluted with 7 ml of physiological saline solution containing a few drops of heparin (the 1% stock solution of Upjohn heparin was prepared without phenol preservative). The diluted blood was centrifuged at 3,000 rpm for 10 minutes, and the supernate then centrifuged at 9,500 rpm for 10 minutes. The second supernate, designated "plasma," was inoculated into 7 newborn C3H mice; of these, 3 developed leukemia at 5 to 6 months of age, 3 other mice developed bilateral parotid gland carcinomas at 4 to 7½ months of age, and 1 died without signs of either tumors or leukemia at 6½ months of age.

Fifth passage. One of the leukemic mice from the preceding passage was used as donor. A leukemic cell suspension prepared from mediastinal tumor, liver, spleen, and peripheral glands, was centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. The final supernate was inoculated into 5 newborn C3H mice. All developed generalized leukemia at 3 to 7 months of age.

Sixth passage. The fifth passage leukemia was carried by cell-graft through 3 successive transplantations into adult C3H mice. The leukemic C3H mouse from the third transplantation was then used as donor from which a leukemic cell suspension was prepared. After centrifugation at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes, part of the final supernate was passed through Selas, porosity 02, filter candle. Three newborn C3H mice were inoculated with the filtrate and all developed leukemia at 3 months of age. Nine newborn mice (3 litters) were inoculated with the 9,500 rpm supernate; 8 developed leukemia at 2½ to 6½ months of age, and 1 is still in good health at 8 months of age.

Seventh passage. One of the leukemic males from the sixth passage with filtrate-induced leukemia was used as donor; liver, spleen and mesenteric tumor were ground, and centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 5 minutes. Part of the final supernate was passed through a Selas, porosity 02, filter candle. Six C3H litters were inoculated as follows: 19 newborn mice were inoculated with the filtrate, (13 subcut., 2 intraper. and 4 intracran.) and all developed leukemia at 2½ to 3½ months of age. Seven newborn mice were inoculated subcutaneously with the 9,500 rpm supernate, and all developed leukemia at 3 months of age.

Alternate seventh passage. Two leukemic females from the sixth passage were used as donors. Leukemic cell suspensions, prepared separately, were centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. Part of the second supernate was passed through Selas, porosity 02, filter candles. Two extracts were prepared. The first extract was inoculated into 2 C3H litters, as follows: 2 newborn C3H mice were inoculated with the 9,500 rpm supernate, and both developed leukemia at 1 month of age. Five newborn C3H mice were inoculated with the filtrate, and all developed leukemia (2 at 2 months, 1 at 3, and the remaining 2 at 5 and 5½ months of age). The second extract, a filtrate, was inoculated into 3 newborn C3H mice, and all developed leukemia at 2½ months of age.

Eighth passage. Several extracts have been recently prepared from seventh-passage-donors, and inoculated into newborn C3H mice. Only one of these experiments has been completed: an extract prepared from a 2-month-

old leukemic female of the preceding passage was centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 5 minutes. The final supernate was inoculated into 13 newborn C3H mice (5 litters), and all developed leukemia at 3 to 3½ months of age.

Preservation of leukemic agent at –70°C. Leukemic cell suspensions prepared in the usual manner from livers, spleen, mesenteric and mediastinal tumors from leukemic donors were placed in glass ampoules; after sealing, the glass ampoules were quickly frozen in carbon dioxide ice, then placed in metal boxes surrounded by blocks of carbon dioxide ice in an ice chest, and preserved at –70°C. The extracts proved active after preservation at –70°C for periods tested thus far up to 4½ months. Defrosted and inoculated into newborn C3H mice, such extracts induced either leukemia, parotid gland tumors, and/or subcutaneous sarcomas, with no appreciable prolongation in latency period. The following experiment will serve as an example: On Apr. 18, 1956, a 9½-month-old Ak female, No. 189, which developed spontaneous leukemia, was sacrificed, and from leukemic organs a cell suspension of 20% concentration was prepared, then placed in an ampoule, sealed, and frozen at –70°C. After 53 days, the ampoule was removed and defrosted in tap water within a few minutes. The ampoule was then opened, its contents aspirated with syringe, placed in centrifuge tube, and centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. The second supernate was inoculated into a C3H litter, consisting of 6 mice; 5 developed leukemia at ages varying 4½ to 7 months.

That the leukemic agent could also be preserved at –70°C as a cell-free extract, was evident from the following experiment: On Aug. 21, 1956, a cell suspension prepared from a leukemic sixth-passage-donor was centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. Three ml of the final 9,500 rpm supernate were then sealed in an ampoule, and frozen in carbon dioxide ice at –70°C. After 46 days, the ampoule was defrosted within a few minutes, and the extract, without any further treatment, was injected into 2 newborn C3H litters. Five mice were inoculated in the first litter, and all developed leukemia at 3 to 3½ months of age. Three mice were injected in the second litter, and 2, thus far, have developed leukemia at 3½ months of age.

TABLE I. Results of Inoculation of Cell-Free (Centrifuged or Filtered) Extracts from Leukemic Passage A Strain into Newborn* C3H Mice.

Passage No.	No. extr. inoc.	No. litters inoc.	No. mice inoc.	No. dev. leukemia	Avg age leukemia dev. (mo)	Leukemia incidence (%)
1†	1	1	7	6	6	86
2	1	1	3	3	3	100
3	1	1	5	2	7	40
4	1	4	7	3‡	6	43(86)‡
5	1	2	5	5	4½	100
6	3	5	12	11	7	92
7	4	10	37	37	3	100
8	1	5	13	13	3	100

* Most mice were < 12 hr old, many < 6 hr, none > 16 hr at time of inoc.

† First extract prepared from 7½-mo-old Ak female with spontaneous leukemia.

‡ Three additional mice in this group developed bilateral parotid tumors at 5 mo of age. Total incidence of leukemia and tumors, 86%.

In another experiment, a leukemic filtrate prepared on Aug. 21, 1956 from a 6th passage donor, was sealed in an ampoule, frozen at –70°C, and then kept in carbon dioxide ice for 103 days. On Dec. 4, 1956, the ampoule was defrosted within a few minutes in tap water, and its contents, without any further treatment, inoculated into a newborn C3H(f) litter. Of 6 mice, 3 have already developed leukemia at 2½ months of age; the remaining 3, now less than 3 months old, are still in good health.

Discussion. During the course of recent experiments, the observation was made that one line of the leukemic agent, that originated in a leukemic Ak mouse, and has been since carried through several successive cell-free passages, induced consistently a high incidence of leukemia, following inoculation into newborn C3H mice. A standardized method of preparing the extract as either a 9,500 rpm supernate, or Selas, porosity 02, filtrate, was adopted; such extracts were inoculated into newborn C3H mice, usually by subcutaneous route, occasionally intraperitoneally or in-

tracranially. Most of the inoculated mice developed acute, generalized, lymphatic leukemia at 2½ to 3½ months of age (Table I). Filtered extracts appeared as potent as centrifuged (9,500 rpm) supernate; subcutaneous, intraperitoneal and intracranial routes of inoculation appeared equally effective. Furthermore, the potency of the agent did not appear to be impaired by preservation at –70°C for 46 days to 4½ months.

The results thus far obtained with the mouse leukemia passage agent, could be compared to studies with the Rous sarcoma virus recently reported by Bryan and his associates (8). At first, when the filterable chicken tumor was discovered by Rous(9), it was not always possible to duplicate cell-free transmission using any tumor-carrying-chicken as a donor for the preparation of the filtrate. Gradually, it has been learned that different tumors contain different amounts of infective virus; cell-free passage of the Rous sarcoma virus from one host to another gradually increased the potency of the extracts. It is now possible to induce sarcomas in chicks with cell-free extracts in 100% of cases after a short latency of only 1 to 2 weeks(8). Ampoules containing the chicken tumor agent can be kept at –70°C and used as needed for inoculation of 3-week-old chicks of a susceptible strain. With chicken sarcoma and chicken myeloblastosis(10), the selection of a susceptible strain of hosts for inoculation of the virus is as important as in mouse leukemia(4). It is equally important, however, to use potent extracts for inoculation.

It should also be emphasized that the cell-free, centrifuged or filtered extracts, prepared from the highly potent passage strain A of mouse leukemia here reported proved to be consistently pathogenic only when inoculated into newborn C3H mice. These mice were in most instances less than 12, never more than 16 hours old at time of inoculation. In each experiment, young adult (1½ to 3 months old) C3H mice have been also routinely inoculated. Thirty-five filtered extracts were prepared, each from a different leukemic donor, and inoculated into a total of 116 young adult (approximately 2-month-old) C3H mice. Only 4 out of 94 adult C3H mice inoculated with the leukemic filtrates intraperitoneally (0.5 to 1 ml each), and 1 out of 22 inoculated intracranially (0.1 to 0.2 ml each), developed leukemia.

Summary. 1. A strain of a filterable mouse leukemia agent has been developed from a spontaneous Ak leukemia through several successive cell-free inoculations of newborn C3H mice. 2. This agent induced acute, generalized leukemia within 2½ to 3½ months in 40 to 100% of the inoculated newborn C3H mice (Bittner substrain). 3. Newborn mice had to be inoculated; adult C3H mice were with few exceptions resistant to inoculation of the cell-free agent. 4. The leukemic agent could be preserved at –70°C from 46 days to 5½ months, with no apparent loss of infectivity.

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Antibacterial Properties of Orinase in Infected Mice. (23081)

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The search for an orally active agent effective in treatment of diabetes mellitus has been carried on for many years. Recently, a group of synthetic sulfonamides has been reported to be capable, when administered orally, of reducing blood sugar concentrations in clinical diabetes(1-5) as well as in experimental animals(5-7).

While continuous administration of any physiologically active drug presents the obvious problem of toxicity, the constant use of a sulfonamide, even though not a sulfanilamide-like compound,* presents another aspect of toxicity not commonly encountered, *viz.*, the effect of the compound on bacterial flora of the host. This question is particularly pertinent in the diabetic individual in view of his increased susceptibility to infection. It therefore appeared advisable to investigate the antibacterial properties of some of the newly discovered antidiabetic agents. In this paper the antibacterial properties of 2 compounds having hypoglycemic activity, 1 - (p-methylphenylsulfonyl)-3 - butylurea or Orinase† and 1-(p-aminophenylsulfonyl)-3-butylurea, are compared with a commonly used triple sulfa mixture.

Methods. Male white mice (CF-1), 18-20 g, were selected at random from animal pools. Technics for establishing mouse infections, maintenance of cultures, etc., have been described(8,9). Freshly prepared stock suspensions in aqueous 0.25% Methocel‡ were prepared at beginning of each experiment and diluted so that the desired dose was contained in 0.2 ml when administered subcutaneously, or in 0.5 ml when administered by oral intubation. The triple sulfa mixture was prepared by thoroughly mixing in 1:1:1 ratio sulfadiazine, sulfamerazine and sulfamethazine. After blending, the mixture was considered an entity and used as the other two compounds. Preliminary work in non-infected animals demonstrated that subcutaneous doses as high as 400 mg/kg/day and oral doses as high as 800-1000 mg/kg/day of the sulfonamide compounds could be safely given to mice at daily intervals over a period of 6 days without gross signs of toxicity. The infecting dose and the drug were administered so that the drug was given immediately following the infecting dose and continued at daily intervals for 6 days. On the seventh day the surviving animals were sacrificed. Evaluation of activity was based on median protective dose (CD_{50}) as calculated by the method of Reed and Muench(10), and the standard error calculated as described by Miller and Tainter (11).

Results. Table II summarizes our data of the comparison of 1-(p-methylphenylsulfonyl)-3-butylurea with 1-(p-aminophenylsulfonyl)-3-butylurea and the triple sulfa mixture in infected mice. The failure of 1-(p-methylphenylsulfonyl) - 3 - butylurea to protect mice infected with lethal challenges of 10 different organisms is quite clearly demonstrated. None of the infected groups survived in statistically significant numbers in those instances where 1-(p-methylphenylsulfonyl)-3-butylurea was given via either oral or subcutaneous routes. This is in contrast to 1-(p-aminophenylsulfonyl)-3-butylurea which possesses a high degree of antibacterial activity in all infections studied with the exception of *Pseudomonas aeruginosa* (Table I). The latter compound is inferior to the triple sulfa mixture.

It is felt that the failure of 1-(p-methylphenylsulfonyl)-3-butylurea to protect infected mice was due to a true lack of antibacterial activity and that the animals did not die of cumulative toxicity produced by infection superimposed upon a near lethal hypoglycemia caused by a large dose of 1-(p-methylphenylsulfonyl)-3-butylurea. If death

* Unpublished.

† Orinase is the Upjohn trade name for 1-(p-methylphenylsulfonyl)-3-butylurea.

‡ Dow Chemical Co., Midland, Mich.

TABLE I. Comparison of *In Vivo* Antibacterial Activities* of Orinase, 1-(p-Methylphenylsulfonyl)-3-Butylurea, 1-(p-Aminophenylsulfonyl)-3-Butylurea, and a Triple Sulfa Mixture.

Organism	1-(p-aminophenylsulfonyl)-3-butylurea		Orinase		Triple sulfa	
	Subcut.	Oral	Subcut.	Oral	Subcut.	Oral
Streptococcus hemolyticus	66.1	54.3	>400	>600	37	10
Diplococcus pneumoniae I	353	236	"	"	114	121
Micrococcus aureus	100	50	"	"	18	10
Pasteurella multocida	55.1	46.8	"	"	14	10
Klebsiella pneumoniae	327	200	"	"	45	80
Proteus vulgaris	31.2	20.2	"	"	2.7	2.1
Pseudomonas aeruginosa	>500	395	"	"	31.4	28.3
Salmonella typhi	12.6	12.6	"	"	13	10
Salmonella paratyphi B	70.7	64	"	"	2.5	4.2
Escherichia coli	100	83	"	"	13.5	9.3

* Expressed as median protective dose in mg/kg/day.

TABLE II. Effect of Glucose* on Antibacterial Activities of 1-(p-Aminophenylsulfonyl)-3-Butylurea and Orinase, 1-(p-Methylphenylsulfonyl)-3-Butylurea in *S. hemolyticus* Infected Mice.

Log dil'n of challenge dose	Orinase†		1-(p-aminophenylsulfonyl)-3-butylurea		Control‡	
	Mort. rate		Mort. rate		Mort. rate	
	With glucose	Without glucose	With glucose	Without glucose	With glucose	Without glucose
4	20/20	20/20	0/20	0/20	10/10	10/10
5	"	19/20	"	"	"	"
6	16/20	17/20	"	"	5/10	8/10
7	5/20	11/20	"	"	"	1/10
8	0/20	0/20	"	"	1/10	0/10
Avg LD_{50}§	6.5	6.9	>4.0	>4.0	6.6	6.4

* Given as 5% in the drinking water. † Admin. orally by direct intubation at 500 mg/kg/day. ‡ Infected but untreated. § Expressed as log of median lethal dose.

had been due to increased susceptibility of the animal, due to a low blood sugar concentration or other forms of toxicity plus the infection, it could be expected that fewer bacterial cells in the challenge, as demonstrated by a higher LD_{50}, would cause death of the animals. Further, if the toxic effect of low blood sugar were countered with glucose administered to the animal, a therapeutic pattern resembling that of 1-(p-aminophenylsulfonyl)-3-butylurea should be observed. In an effort to demonstrate this, the following experiment was designed and carried out.

Two groups of 200 mice were treated with either 1 - (p-methylphenylsulfonyl)-3 - butylurea or 1-(p-aminophenylsulfonyl)-3-butylurea. The 2 groups were further divided into 5 groups of 40 mice, and each group was challenged with *Streptococcus hemolyticus* serially diluted in 10-fold increments. One-half of the animals treated with 1-(p-methylphenylsulfonyl)-3-butylurea was given glucose in drinking water, while the remaining half was given plain water. Appropriate untreated infected controls were used for comparative purposes. The median lethal dose of *S. hemolyticus* for each group was used as a measure of altered susceptibility to the infection.

It can be noted from the data (Table II) that addition of glucose (5%) to the infected animals did not alter susceptibility of the different groups to the *S. hemolyticus* infections. The groups receiving 1-(p-aminophenylsulfonyl)-3-butylurea was protected whether glucose was administered to mice or not, and the susceptibility of mice receiving 1-(p-methylphenylsulfonyl)-3-butylurea was unchanged in the untreated starved controls.

The influence of one bacteriologically active agent upon activity of a second antibacterial agent has been studied in detail by numerous workers. Many types of different ac-

TABLE III. Effect of Orinase, 1-(p-Methylphenylsulfonyl)-3-Butylurea on the Activity of Tetracycline in *S. hemolyticus* Infected Mice, 20 Mice per Series.

Drug level*				Drug level*			
Orinase	Tetracycline	Mort. ratio†	CD_{50}‡	Orinase	Tetracycline	Mort. ratio†	CD_{50}‡
250	.3	19/20	.73 (.53-.93)	None	.3	17/20	.72 (.60-.84)
"	.6	14/20		"	.6	12/20	
"	1.2	1/20		"	1.2	5/20	
"	2.4	0/20		"	2.4	1/20	
"	4.8	"		"	4.8	0/20	

* Expressed in mg/kg/day. † No. dead/total No. ‡ Median protective dose (95% confidence limits).

tions, including synergism and antagonism, have been demonstrated depending upon the antibiotics and upon the proportions. To establish that 1-(p-methylphenylsulfonyl)-3-butylurea does not interfere with antibacterial action of an antibiotic, the compound was administered orally to a group of infected mice being treated subcutaneously with tetracycline§ while a second group of infected mice was treated with the antibiotic alone. The median protective dose of tetracycline was determined both alone and in the presence of 250 mg/kg/day of 1-(p-methylphenylsulfonyl)-3-butylurea and used as the basis of evaluation.

From the results presented in Table III, it is obvious that 1-(p-methylphenylsulfonyl)-3-butylurea does not interfere with the protective action of tetracycline in *S. hemolyticus* infected mice, and that tetracycline therapy could be carried out in the presence of relatively large daily doses of 1-(p-methylphenylsulfonyl)-3-butylurea.

Summary. 1. The chemotherapeutic properties of 2 newly discovered sulfonamide antidiabetic agents have been studied in infected mice and compared to a commonly used triple sulfa mixture. Data demonstrating that 1-(p-methylphenylsulfonyl)-3-butylurea is devoid of any antibacterial activity and that 1-(p-aminophenylsulfonyl)-3-butylurea is active as an antibacterial agent have been presented. Lack of antibacterial activity of 1-(p-methylphenylsulfonyl)-3-butylurea can be attributed to absence of the para-amino group. This para-amino group is present in each of the ingredients in the triple sulfa mixture as well as other sulfanilamide-like compounds. 2. It has been demonstrated that failure of 1-(p-methylphenylsulfonyl)-3-butylurea to protect infected mice was due to a true lack of antibacterial activity, and death of test animals was not attributed to toxicity. 3. Large daily doses of 1-(p-methylphenylsulfonyl)-3-butylurea have been shown not to interfere with therapeutic activity of tetracycline in infected mice.

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Some Effects of N-Nitrosodimethylamine on Rabbit Kidney Function. (23082)

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N-nitrosodimethylamine (NNDMA) has become of interest as an intermediate in the manufacture of asymmetrical dimethyl hydrazine, used as a component of rocket fuels. One of 2 laboratory workers exposed accidentally to this compound died of "acute terminal passive congestion" of the kidneys(1). Other cases have shown toxic effects on the liver(2,3,4). Animal experiments have shown degenerative and malignantly proliferative changes in liver(3,5). Because fatally poisoned animals may die within a few hours and because hepatectomized animals may be expected to survive for up to 40 hours(6), work was undertaken to see whether NNDMA has other significant toxic effects. The early report implicating the kidney in NNDMA toxic effects(1), and our own observation that the material caused variations in urine output in dogs, suggested that kidney effects might be a part of the toxic mechanism of this compound.

Materials and methods. Rabbits of mixed breed and sex were used for kidney function studies in which inulin and p-aminohippuric acid (PAH) clearances were measured(7). The animals were lightly anesthetized with pentobarbital and infused continuously with physiological saline solution containing inulin 2.5 mg/ml and PAH 14.3 mg/ml, at 1 ml/min. Blood samples were taken from a deep femoral artery, and urine flowed through polyethylene catheters in the ureters. Urine was collected at 30-minute intervals and corresponding blood samples were taken at midpoints of the intervals. Urine and blood were analyzed for inulin(8) and PAH(9).

Rabbits which had been injected intravenously with NNDMA (10 mg/kg or ⅔ approximate LD_{50}) 18, 45 and 65 hours previously were anesthetized and prepared as described above. Two or 3 animals were studied at each time, yielding 6 experimental periods each. Values obtained from poisoned animals were compared with those from 7 animals which received no NNDMA, using the "t" test(10,11).

Results. Preliminary experiments on short-term effects of 60 mg/kg of NNDMA on renal function showed no significant changes from control values, with the possible exception of a decrease in fPAH(7) 2 hours after NNDMA was given.

Longer term studies of kidney function are presented in Table I. It is apparent that 18 hours after injection of NNDMA, urine flow was significantly decreased. There was also a significant decrease in clearance of PAH. While it is questionable that the simultaneous decrease in mean glomerular filtration rate (inulin clearance) is real, there is little doubt that the tubular epithelium transfers less PAH into the tubular lumina at 18 and 45 hour intervals.

The net result of the kidney studies, then, is that NNDMA (60 mg/kg) until 2 hours after injection has no clearly adverse effects upon renal function, but that there may be decrease in tubular function beginning at this time. Loss of tubular function is definite 18 hours after administration of smaller doses of NNDMA (10 mg/kg), and continues through the 45 hour period. Some recovery from the impairment is evident by 65 hours. Filtration is not significantly impaired, but a minimal decrease in filtration rate may be present also.

Urine flow decreased significantly at 18 (by 67%) and 45 (by 76%) hours, and this was simultaneous with depressed tubular excretion of PAH. Since filtration had decreased by only 47% and 58%, respectively, at these 2 times, the rest of the decrease in urine flow must occur by increased passage of water from the tubular lumina into the peritubular capillaries. We observed that capsules of kidneys of poisoned animals are usually tense. This observation suggests that oliguria in NNDMA poisoning may result in part from increased intracapsular pressure

TABLE I. Long Term Effects of NNDMA on Rabbit Kidney Function, Dose 10 mg/kg, I.V.

Hr after NNDMA	N	Urine flow ml/min. ± S.E. and (P)*	Inulin clearance ml/min. ± S.E. and (P)*	PAH clearance ml/min. ± S.E. and (P)*	fPAH ± S.E. and (P)*
0	42	.76 ± .067	3.64 ± .714	18.39 ± 2.450	9.61 ± 2.645
18	18	.25 ± .063 (.001)*	1.92 ± .602 (.1)	4.60 ± .421 (.001)	2.09 ± .425 (.05)
45	12	.18 ± .024 (.001)	1.52 ± .248 (.1)	2.19 ± .611 (.001)	2.75 ± .803 (.1)
65	12	.79 ± .137 (.8)	3.22 ± .475 (.1)	13.33 ± 1.660 (.2)	4.01 ± .645 (.2)

* P = Probability of chance difference from controls.

N refers to No. of 30-min. clearances measured. Value fPAH(7) represents tubular excretion rate of PAH in terms of glomerular filtration rate, and was calculated independently for each clearance measurement.

with increased back-diffusion of water across the tubular epithelium. Also, when kidneys of animals used in long-term studies were sectioned, stained and examined microscopically, vascular congestion, particularly in the area of the collecting tubules, was evident in poisoned animals as compared with controls. Such congestion could contribute to increased intracapsular pressure and oliguria(12), and appeared correlated in degree with functional changes. Livers of experimental animals showed central zone damage as reported by other investigators(3,4).

Preliminary experiments indicated that circulatory, respiratory, and autonomic nervous systems are not implicated to any important extent in acute death resulting from administration of NNDMA. The principal causal factor in such deaths has been recognized generally as liver damage(2,3,4).

Summary. 1. The present studies have shown that, in addition, significant loss of kidney function can result from exposure to this material. Such kidney debilitation could be of considerable consequence if it occurred together with severe liver damage. The time of occurrence of maximal kidney effects correlates well with time of death after NNDMA, as well as with that of rise of blood NPN in rabbits and in dogs. 2. No histologically demonstrable changes in functional kidney cells were evident in these animals nor in those examined by other investigators(3,4). However, other investigators found local vascular congestions in liver, lungs and intestine. The present experiments indicate this to be true in kidneys which show significant loss of function. The suggestion(3) that an outstanding property of NNDMA is its affinity for vasculatures of liver, lung and intestine may also apply to vasculature of kidney.

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Serum Cholinesterase Activity in Thymectomized Rats. (23083)

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Hawkins *et al.*(1) found an inverse relation between thyroid and serum cholinesterase activities in the rat. Thyroidectomy produces regression of the thymus(2,3,4). Thyroid extract causes hypertrophy of the thymus, although an excess leads to atrophy(2). Then would thymectomy cause a change in esterase activity?

Methods. Fourteen young (50-60 g) female albino rats of the Wistar strain were thymectomized under light ether anesthesia according to the technic of Segaloff(5). Fourteen control rats were anesthetized and sham-operated; the incision was held open for the average time necessary to perform a thymectomy. Three weeks after the operations the animals were bled. Serum esterase activity was measured by a modification of the optical method developed by Kalow(6) (serum dilution, 1 to 100; temp. 40°C; substrate, 5 x 10^{-5}M benzoylcholine chloride; wavelength, 240 mμ; duration of measurement, 15 min.). Results were expressed as shift in absorbance x 1,000.

Results. In the control rats the mean esterase activity was 37.5 units, in the thymectomized, 37.6 units.

Summary Although the thyroid gland influences both the status of the thymus and serum cholinesterase activity, thymectomy alone has no influence on serum cholinesterase activity.

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Effect of Incubation upon Folic Acid Content of Eggs. (23084)

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The effect of incubation of eggs upon content of most of the B-vitamins has been determined. Snell and Quarles(1) found no change in the level of riboflavin or pantothenic acid in eggs throughout one period of incubation. Biotin concentration remained constant, or decreased slightly, while inositol and nicotinic acid were found to be synthesized by the embryo. Okamoto(2) found the concentration of thiamin to remain constant. Cielens(3) reports that 53-67% of choline is lost during incubation. Folic acid has apparently not been studied.

Since germfree rats have been found to synthesize vit. B_9 (folic acid and citrovorum factor) while they require biotin and other B-vitamins(4), it is of special interest to determine the change in folic acid content of the incubating egg (which is usually germfree). Evans *et al.*(5) studied the effect of cold storage on folic acid content of eggs and Ebert (6) reported on development and role of p-aminobenzoic acid (a component of folic acid) in the early chick embryo.

Methods. Four dozen eggs were procured

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TABLE I. Folic Acid Level of Eggs at Different Stages of Incubation.

Group	Incubation, wk	No. eggs	Mean folic acid/egg, μg (± stand. dev.)
I	0	12	6.5 ± 2.68
II	1	11	5.5 ± 2.02
III	2	9	5.1 ± 1.99
IV	3	7	5.6 ± 1.64

the same day from a flock of hens (White Vantress males crossed with White Rock hens from Smith Hatchery, Columbia, Mo.) receiving identical diet. The eggs were divided into 4 groups of one dozen each, they were numbered according to group and after formaldehyde treatment they were incubated 0, 1, 2 and 3 weeks respectively by a commercial hatcher. The eggs were then assayed by a microbiological method(7) employing *S. fecalis,* A.T.C.C. No. 8043. Powdered hog kidney† was used as a source of folic acid conjugase in view of data presented by Sreenivasan *et al.*(8), Olson *et al.*(9) and Bird *et al.*(10). Commercially‡ prepared growth agar and assay media were used.

Results. A high individual variation was found between eggs within each group despite the attempt to obtain relatively uniform eggs. As shown in Table I the mean folic acid level per egg for each group falls within one standard deviation of variation from the means of all the other groups. These data add to emerging metabolic patterns which show why a chick requires a dietary source of folic acid while a rat does not(11). Since there appears to be no increase in total quantity of folic acid in an egg, as the embryo develops, isotopic studies are needed to determine whether the nutritional requirement of the chick for folic acid is due to complete absence of a synthetic mechanism or simply the presence of a requirement greater than synthesis under a given set of conditions.

Conclusion. No change in concentration of folic acid was found in eggs during incubation for 3 weeks.

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Minimal Vitamin Requirements of Rabbit Fibroblasts, Strain RM3-73. (23085)

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Isolation of a subline of rabbit fibroblasts, designated RM3-73, from the established stock strain RM3-56(1) has been described.‡ Strain RM3-73 appears to be less fastidious than RM3-56 since it can be propagated serially in a medium containing a small quantity of dialyzed serum as the only undefined component, whereas RM3-56 requires both whole serum and chick embryo extract for continuous proliferation. As a basis for comparing nutritional requirements of the two strains, it was of interest to determine some of the factors required by RM3-73. This strain is particularly suitable for nutritional studies since the cells can be propagated for indefinite periods in a medium which contains a minimal quantity of undefined constituents. Thus, some of the restrictions which are imposed by inadequate basal media are eliminated.‡ This report describes the vitamins required by strain RM3-73 in a medium containing 2% dialyzed serum.

TABLE I. Composition of Medium 73.

Compound*	Conc., mM	Compound	Conc., mM
Alanine	1.00	Calcium chloride	1.8
α-Aminobutyric†	.12	Magnesium chloride	.8
Arginine	.20	Potassium chloride	5.4
Asparagine	.07	Sodium bicarbonate	26.2
Aspartic	.11	Sodium chloride	116.2
Cystine	.05	Sodium monobasiephosphate	1.2
Glutamic	.11	Glucose	5.6
Glutamine	2.00	Glucuronic acid	.05
Glycine	.30	Orotic acid	.32
Histidine	.05	Phenol red	.01
Hydroxyproline	.05	Biotin	.005
Isoleucine	.25	Choline	.01
Leucine	.25	Folic acid	.005
Lysine	.25	Nicotinamide	.005
Methionine	.10	Pantothenic acid	.005
Ornithine	.16	Pyridoxal	.005
Phenylalanine	.15	Riboflavin	.0005
Proline	.13	Thiamin	.005
Serine	.25	Vit. B_{12}	.0001
Threonine	.25		
Tryptophan	.03		
Tyrosine	.10		
Valine	.25		

* Medium also contains 100 units of penicillin G and 50 μg of streptomycin sulfate/ml and 2% (v/v) dialyzed horse serum.

† DL-α-aminobutyric acid; all other amino acids are of L- configuration.

TABLE II. Effect of Omitting Vitamins Separately from Medium 73 on the Proliferation of RM3-73.

	Proliferation index after 7 days*			
	Passage			
Vit. omitted	1st	2nd	3rd	4th
Folic acid	6.6	1.0	D†	
Nicotinamide	.8	D		
Pantothenic acid	4.4	.8	D	
Pyridoxal	6.3	6.4	6.1	5.4
" ‡	4.1	1.6	.5	D
Riboflavin	.5	D		
Thiamin	7.6	7.0	3.3	D
Biotin	9.3	7.0	7.2	7.9
Choline	7.2	7.3	6.2	8.1
Vit. B_{12}	8.2	8.4	7.6	8.3
None	7.6	7.2	8.3	7.5

* Avg value for 2 experiments.

† D = Extensive degeneration of cells and counts not performed.

‡ Alpha-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, and proline in addition to pyridoxal omitted from medium 73.

Methods. Stock cultures of RM3-73 were propagated on glass in medium 73 (Table I) according to methods described previously (1). Each experimental medium was evaluated by propagating the cells serially in a group of flasks (1 to 4 T60 and 4 T15 flasks). The medium was replaced on the third and fifth days, and subcultures were prepared at intervals of 7 days by the use of trypsin.‡ The degree of cellular proliferation for each 7-day interval was determined by the nuclear counting procedure.‡

Results. The results obtained when vitamins of medium 73 are omitted separately from the medium are summarized in Table II. The degree to which rate of proliferation de-

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TABLE III. Proliferation of Strain RM3-73 as a Function of Concentration of Individual Vitamins.

Vitamin	Passage No.	Vitamin conc., mM × 10^{-3} 10	1	.1	.01	.001	mM × 10^{-3} permitting optimal growth
		Proliferation index*					
Folic acid	3-4	7.1	9.3	D†			1
Nicotinamide	2-3	7.0	6.3	3.8	D		1
Pantothenate	2-3	7.1	7.5	7.4	3.3	D	.1
Pyridoxal‡	3-4	4.1	5.0	5.2	2.1	D	.1
Riboflavin	2-3		7.1	8.9	3.3	D	.1
Thiamin	4-5	6.9	7.2	7.6	4.4	D	.1

* Avg values for passages indicated in Column 2.
† D = Cells degenerated.
‡ α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine, and proline in addition to pyridoxal omitted from medium 73.

clines in each successive culture passage is a function of the particular vitamin omitted, and this appears to be unrelated to concentration of that vitamin which permits optimal proliferation (see Table III). On continued serial propagation of RM3-73 in the absence of one of the essential vitamins, the cells gradually degenerate (compare Fig. 1, 2, and 3 with Fig. 4) until lysis occurs. It will be noted in Table II that pyridoxal presents a special case since, except for a modest decrease in the rate of proliferation, there is no apparent requirement for this vitamin when omitted separately from medium 73. On the other hand, when α-aminobutyric, asparagine, aspartic, glutamic, glycine, hydroxyproline, ornithine and proline are omitted in addition to pyridoxal, the cells fail to proliferate for more than 2 passages. It is also of interest that pyridoxal is the only vitamin which inhibits proliferation when employed in 100 times the concentration permitting optimal growth (Table III). This group of so-called accessory amino acids which exerts a sparing action on the requirement for pyridoxal is not required for the continuous proliferation of RM3-73 in the presence of the vitamin. The group of accessory amino acids are effective however in increasing rate of proliferation of RM3-73 in the presence of pyridoxal as indicated by the results presented in Table III. The data in Table II which indicate that biotin, choline, and vit. B_{12} are not essential were substantiated by propagating RM3-73 cells for 15 passages in a modified medium 73 lacking these components. In a similar experiment, it was demonstrated that neither glucuronic nor orotic acid is required by RM3-73.

Growth response of RM3-73 as a function of the concentration of each of the essential vitamins is illustrated in Table III. Determinations of this type are complicated by the fact that one or more passages are usually required to deplete the cells of the vitamin and its conjugates when suboptimal levels are supplied in the medium. The data presented in Table III (after 2 to 5 passages in experimental media) are thus representative of the responses anticipated on continued proliferation. This was substantiated in part by experiments employing suboptimal concentrations (1 x 10^{-5} mM) of pantothenic acid and riboflavin. Under these conditions, the level of proliferation indicated in Table III was maintained for at least 8 passages.

Discussion. The foregoing data indicate that folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin are essential for the continuous proliferation of rabbit fibroblasts strain RM3-73. It is apparent that these represent only the minimal requirements of this strain of cells, since additional vitamins may be contributed by unidentified factors supplied by the dialyzed serum. The degree to which the growth rate of RM3-73 declines with each successive culture passage in the absence of an essential vitamin (Table II) is indicative of the rate at which the cells are depleted of the vitamin and its derivatives. This type of response to a nutritional deficiency illustrates the importance of supplementing information obtained in short-term experiments (5-10 days without preparing subcultures) on cell culture nutrition with data procured in studies which are conducted for a sufficient period to permit serial cultivation in the experimental media. In view of the extensive degenerative changes observed when cells are propagated in the absence of an essential vitamin, it is considered that the procedure employed in these studies is somewhat more reliable than that of de-

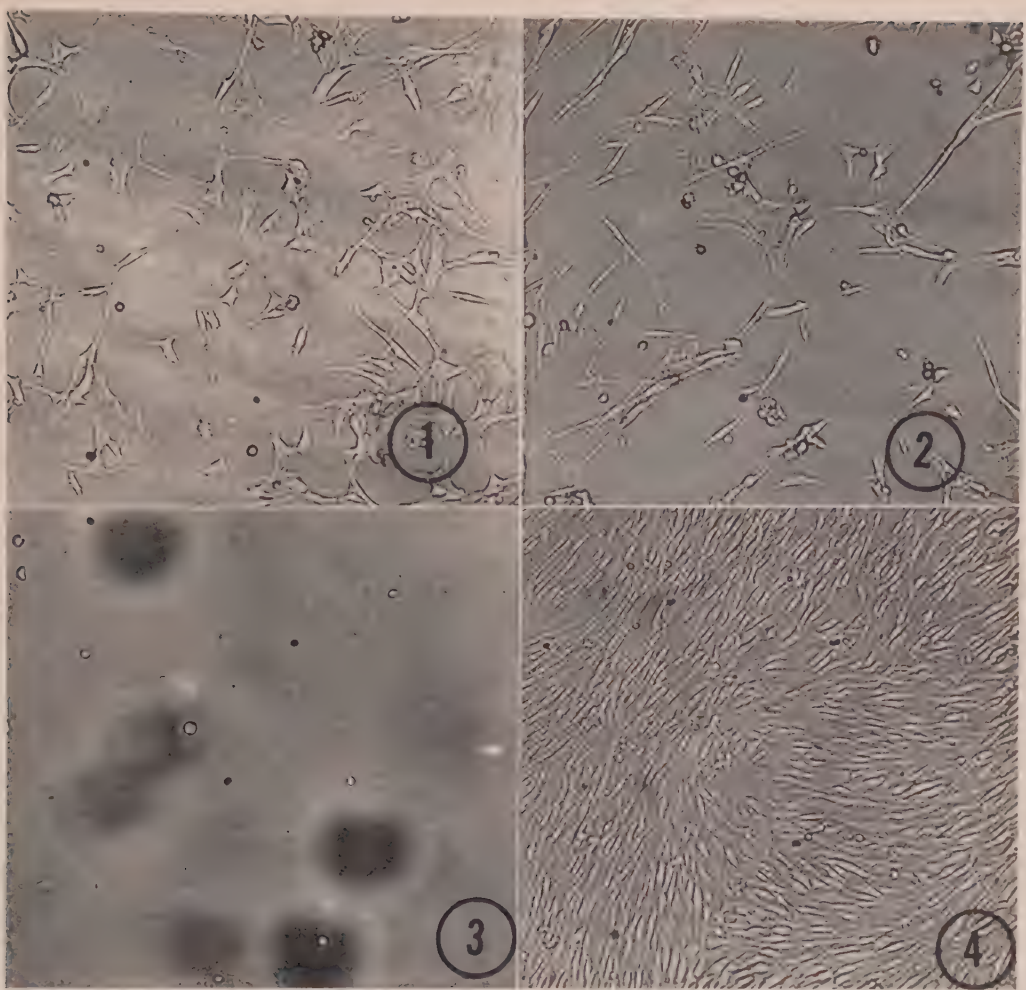

FIG. 1. RM3-73 cells after 7 days in absence of nicotinamide.
FIG. 2. " " " " " " " " riboflavin.
FIG. 3. " " " 14 " " " " pantothenic acid.
FIG. 4. A typical 7-day culture of RM3-73 in medium 73.

termining the concentration of vitamin required to restore the active proliferation of severely depleted cells(2).

RM3-73 resembles certain microorganisms(3,4) in that its requirement for pyridoxal is replaced for at least 5 passages (representing approximately 3 x 10^4 fold increase in number) by a group of eight amino acids which are otherwise not required for the continuous propagation of this strain of fibroblasts under the conditions employed. The data are consistent only with the interpretation that amino acids reduce the requirement for pyridoxal to a minimal level since the dialyzed serum added to the medium may not be entirely free from the vitamin.

The number of vitamins required by RM3-73 is less than that required by other permanent strains of cells which have been studied under similar conditions. Strains L and HeLa require choline(2) in addition to those indicated for RM3-73 (Table III) and several strains require i-inositol(5). Human fibroblasts, strain U12-79, require both choline and i-inositol.§ It is of considerable interest in this connection, that RM3-73 was isolated

§ Unpublished experiments.

by a process of selection from strain RM3-56 which requires whole chick embryo extract and horse serum in addition to the defined components of medium 73.‡ This suggests the possibility that additional vitamins are required for the RM3-56 strain of rabbit fibroblasts.

Summary. A strain of rabbit fibroblasts designated strain RM3-73 requires folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamine for continuous proliferation in a medium containing 2% (v/v) dialyzed horse serum. The concentration of each vitamin which permits optimal proliferation was determined by propagating the cells serially in media containing graded concentrations of the compound. A group of eight amino acids which are not required for serial propagation in the presence of pyridoxal replaced the requirement for this vitamin under experimental conditions employed.

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Viremia in Coxsackie B Meningitis. (23086)

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Viruses of the Coxsackie B group have been frequently isolated from the stools and less frequently from the throat and the spinal fluid of aseptic meningitis patients. However, demonstration of viremia has received only a passing mention in the literature(1,2). The present report describes isolation of a Coxsackie B2 virus from the serum of a patient 3 days after onset of a febrile illness, but 5 days prior to the onset of frank meningitis.

The patient, a 36-year-old male physician engaged in virus research, became ill in November 1955 while investigating an outbreak of a disease characterized by fever, dizziness, chest pain, myalgia and sometimes nuchal discomfort. His studies included examination of patients, collection and testing of blood, pharyngeal and fecal specimens, some of which were found to contain Coxsackie B2 virus.

Methods. Monkey kidney cultures prepared by a modified Youngner technic(3) were used both for virus isolation and neutralization tests. The cells were grown in the lactalbumin hydrolysate-calf serum medium recommended by Melnick(4) and after 4 days of incubation were changed to a maintenance medium in which the concentration of the calf serum was decreased by one half. The human amnion cell cultures were prepared according to a procedure described by Takemoto and Lerner(5). In virus isolation attempts, 0.3 ml of undiluted sera and spinal fluid were added to several tubes each; the throat swabs in Hanks' solution were first treated with 500 u penicillin, 500 μg streptomycin, and 100 u mycostatin per ml; the stools were emulsified to make 10% suspension in Hanks' solution and treated with 1,250 u penicillin and streptomycin both before and after centrifugation at 8,000 rpm for 30 minutes. Serum-virus neutralization tests employed mixtures of equal volumes of appropriate serum and virus dilutions representing 100 tissue culture infectious doses which after 1 hr. at room temperature were inoculated into culture tubes. All sera used in neutralizations, including serial specimens from the patient, were heated at 56°C for 30 minutes.

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TABLE I. Clinical Course and Coxsackie B2 Isolations in Tissue Culture and Suckling Mice.

Date	Clinical course	Specimen	Virus isolations TC	Virus isolations SM
11/12/55	Fever, dizziness, myalgia	None		
13	Same + pleurodynia, nausea	"		
14	Same + headache, anorexia	Throat Blood	Neg. Pos.	Neg. Pos.
15	Some improv'm'nt	None		
16	Same	Stool	"	"
17	Chest pain, backache	None		
18	Same + ? stiff neck	"		
19	Frank clinical meningitis*	Throat CSF	Neg. "	Neg. QNS
22	Some improv'm'nt	Stool	Pos.	ND

* CSF-76 cells (50% PMN), protein 52 mg. Nuchal and spine rigidity, fever, headache and vomiting.

Virus isolations in tissue culture were confirmed by intraperitoneal (0.04 ml) and intracerebral (0.02 ml) inoculation of Swiss white suckling mice of less than 24 hr. of age.

Results. Table I summarizes the major clinical manifestations chronologically with the results of virus isolation attempts. The virus was present in the blood but not the throat secretions on the third day of the initial phase of illness. Its titer was found to be 10^2 per ml of serum at that time. Two days later, during the period of relative improvement, the virus was present in the stool at a titer of 10^5 per g of fecal material. At the time of clinical meningitis, the virus was again not found in the throat and could not be isolated from a sample of spinal fluid. During early convalescence the virus was still present to a titer of 10^4 per g of stool specimen.

Presence of the virus in the patient's blood on November 14 was established by repeated isolations from 2 separately frozen vials of the serum. Three of the isolations were accomplished in tubes prepared from different batches of monkey kidney cells. The fourth attempt utilized human amnion cell cultures; the primary tubes did not show cytopathic changes during the 30-day observation period. Aliquots of supernatants harvested at 10, 20, and 30 days and passaged blindly in amnion and monkey kidney tubes generally failed to produce cytopathic effects. The only exception was the 10 day harvest passaged in monkey kidney which after a prolonged incubation period showed passageable characteristic Coxsackie B effect, suggestive of limited survival rather than propagation of the virus in the amnion system. The fifth virus isolation from the serum was directly in 1 day old suckling mice which developed typical experimental Coxsackie B disease. A passageable cytopathogenic agent was re-isolated in monkey kidney tubes from the brain-muscle pool of the 4th suckling mouse passage. Each of the 5 separate tissue culture virus isolates was individually typed as Coxsackie B2.

Table II shows that neutralizing antibodies against Coxsackie B2 prototype virus, as well as the virus strains isolated from the patient's blood and stool, were present in the convalescent serum but not in the "acute" virus-bearing serum or the sera collected 2 and 16 months before the onset of illness.

Discussion. Demonstration of viremia in this patient suggests that it may be a part of pathogenesis of Coxsackie B meningitis. Unfortunately, because serum, pharyngeal, and fecal specimens were not collected daily, it is not possible to correlate the presence of the virus with the development of specific symptoms. However, it appears that viremia either followed or accompanied the onset of fever, myalgia, and pleurodynia, and preceded the development of clinical meningitis. The absence of demonstrable virus in the throat secretions on 2 occasions and its presence in the stool mid-way through the febrile

TABLE II. Titration of Patient's Sera against Coxsackie B2 Viruses.*

Date of serum†	Virus strains: Serum isolate (11/14/55)	Stool isolate (11/16/55)	Prototype B2
7/26/54	<4	ND	<4
9/15/55	"	<4	"
11/14 ‡	"	"	"
12/ 6	64	64	64

* Figures indicate reciprocals of serum dilutions neutralizing 100 $TCID_{50}$.

† All sera were heated at 56°C for 30 min.

‡ "Acute" virus-bearing serum.

illness preceding meningitis suggests that in this patient there was an early locus of viral propagation in the intestinal tract.

Review of the literature disclosed no other findings suggestive of the possible role of Coxsackie B viremia in the pathogenesis of meningitis. Isolation of 4 Coxsackie virus strains from human blood was first reported by Taylor(1) in the course of routine testing of 1,990 specimens from febrile patients in Egypt. Two of these viruses were found to belong to Group A (A3 and A6) and 2 were typed as B2. No definitive data were presented correlating the presence of viremia with the clinical picture(1,6).

Another cursory reference to a single isolation each of Coxsackie A and B viruses from human bloods was made by Bayer and Gear (2). No details were given but apparently all of their patients were admitted with CNS involvement as suspected cases of nonparalytic poliomyelitis. The uncommonness of Coxsackie B viremia after onset of meningitis is attested by the fact that they were able to isolate the virus only once although their group of 200 cases of viral meningoencephalitis included 20 individuals with Coxsackie B viruses in the feces, 9 of whom also had demonstrable virus in the spinal fluid.

It is quite possible that every systemic infection with the Coxsackie B viruses is accompanied by viremia. Elucidation of its role in the pathogenesis of complicating meningitis will require further attempts to isolate the virus from all types of suspected Coxsackie B infections, including those with meningeal involvement at various stages of the disease.

Summary. Coxsackie B2 virus was repeatedly isolated from a serum specimen obtained 5 days prior to the onset of clinical meningitis. The virus was also demonstrated in the stool specimen during the prodromal phase and in convalescence. The convalescent serum contained specific antibodies which were not present either in the virus-bearing serum or in sera collected before the onset of this illness.

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Relationship Between Estradiol and Parathyroid on Retention of Ca^{45} in Bone and Blood Serum of Rats.* (23087)

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In several species the parathyroid glands of female animals have been reported to be heavier than the male of equal body weight. These reports include data on rats(1-4), man (5,6), and Brown Leghorn fowls(7). In rabbits, goats(4) and bantams(8) no sex difference was observed. The reported influence of ovarian hormones on calcium metabolism has been variable. In the rat, estrogen has been reported to increase blood calcium (9,10), or have little or no effect(11-14). In the guinea pig no effect was observed(4). In rabbits, the reports varied from no effect(9, 12,13,15) to decrease in blood calcium(16-18). In dogs, the reports vary from an increase(9,19,20) to no effect(12,21). In cattle a slight decrease in blood calcium was noted(22,23). These variable results may be due to the use of non-physiological doses of estrogen or to other unknown factors. The osteogenetic properties of estrogenic hormones in birds, mice and rats have been reported. A recent review of this literature has appeared(24). Recently, it was demonstrated in rats that after parathyroidectomy females lived longer than males(25). If, in addition to parathyroidectomy, the females were previously ovariectomized, their life span was as short as the males(26,25), and if the males were treated with a small quantity of estradiol, they lived as long as non-castrated females(27).

In the light of variable observations concerning the role of estrogenic hormone in calcium metabolism, it seemed desirable to extend the previous observations using Ca^{45} as a measure of effect of estrogen.

Procedure. Thirty-nine adult male albino rats were divided into 6 groups and treated as indicated (Table I). Prior to and during the experimental period the animals were fed a stock diet. All animals were housed in wide-mouthed glass jars which contained wire screens approximately 2 inches from the bottom and prevented the rats from eating their feces. Estradiol benzoate was injected subcutaneously daily, at a level of 82.5 μg in 0.25 ml of sesame oil. Parathyroid extract was administered subcutaneously daily at a level of 50 U.S.P. parathyroid hormone units. Parathyroidectomized rats were treated for 2 days post-operatively with Penicillin G. Ca^{45}, in the form of $CaCl_2$, (specific activity >0.2 mc/g of Ca) was injected intraperitoneally at total dosage of 2.65 μc/100 g body weight during 4 days. Considering radioactive decay each rat received a total of approximately 20 to 28 mg of calcium/100 g body weight. Throughout all operational procedures of parathyroidectomy or of blood sampling the anesthetic was chloroform. The animals were killed 2 days after last injection by exsanguination from the left carotid artery. After centrifugation of the clotted blood 2 aliquots of 0.5 ml of serum were dried in metal counting cups and radioactivity measured in duplicate. To measure radioactivity in bone, the right femur was carefully cleaned of muscular and tendinous tissue, weighed and completely digested (approximately 24 hours) in 2 ml of hydrochloric acid (1.18 sp. gr., 37% HCl). The digest was filtered through glass wool and the filtrates diluted to 10 ml with distilled water which had been used to wash the digestion flask and digest retained on glass wool. From this solution 3-1 ml aliquots were dried in glass counting cups and the radioactivity of the femur measured in triplicate. Corrections were not made for sample self-absorption.

Results. Mean values in counts per min-

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TABLE I. Radioactivity of Serum and Bones of Male Rats Injected with Ca^{45} for 4 Days.

Group	Treatment	Bone, mean count/min./g (± S.D.)	Serum, mean count/min./ml (± S.D.)
1	Control (8)‡	21168 ± 4143.9	199 ± 42.1
2	Estradiol (8)	20233 ± 3454.8	†344 ± 61.5
3	Parathyroidectomy (6)	*16254 ± 2082.0	174 ± 52.4
4	" + estradiol (7)	17309 ± 3691.6	†300 ± 113.4
5	Parathyroid extract (4)	†12602 ± 1003.6	171 ± 30.1
6	" " + estradiol (6)	*16076 ± 2607.2	257 ± 74.4

* $P < 0.05$. † $P < 0.01$. ‡ No. of animals in group.

ute and their standard deviations (σ) for bone and serum are presented (Table I). The difference in the amount of Ca^{45} found in bone of estrogen-treated and control groups was not significant.

After parathyroidectomy the amount of Ca^{45} present in bone was decreased ($P<0.05$) confirming the results of Talmage *et al.*(28) who reported a significant decrease in rate of calcium turnover in the absence of parathyroids. Although not significant, injection of estrogen tended to increase the uptake of Ca^{45} in parathyroidectomized animals.

Administration of parathyroid extract caused a significant ($P<0.01$) decrease in the amount of Ca^{45} present in bone. Employing autoradiographic technic, Talmage *et al.*(28) similarly found that 24 hours after treatment with parathormone there was a detectable amount of Ca^{45} removed from the bone.

Comparison of Groups 1, 5 and 6 demonstrates that when estrogen is administered to rats receiving parathyroid extract, there was a tendency to inhibit removal, or induce deposition of Ca^{45} in bone. Inhibition of parathormone action on bone demineralization was not complete as seen by the fact that the difference between the controls and group(6) treated with both hormones was still significant ($P<0.05$).

Following treatment with estradiol the amount of Ca^{45} still present in serum at the time the rats were killed was significantly higher than in the control group ($P<0.01$). Although this confirms the findings of Manunta(29) who reported that in rats treated with synthetic estrogens there was a significant decrease in urinary calcium, it is contrary to the results of other workers who found no difference in serum calcium levels of rats similarly treated. Re-examination of the controversial data showed results that were variable but still within the physiological range and non-significant. It is believed that the different results are due to the different procedures used and sensitivity of methods. To test the sensitivity of the method an *in vitro* technic was employed. To normal blood serum sufficient Ca^{40} and Ca^{45} was added to raise the calcium level 1 mg. The radioactivity of serum was 1.216 $\mu\mu$c/ml. With the apparatus used this gave 180 counts/minute/ml. It is evident that this method allows measurements of slight differences in serum Ca^{45} levels.

In the parathyroidectomized - estrogen-treated group Ca^{45} was higher in blood and the differences statistically different from control and parathyroidectomized groups. The latter group had a lower amount of serum Ca^{45}, but it was not statistically different from the controls, probably because of a continuous removal from the bone.

It has been known and recently reaffirmed (28,30) that parathyroid extract causes a rise in serum calcium. Under the same treatment (Group 5) a non-significant decrease was found. This is not interpreted as a lack of hypercalcemia, but as a prompt excretion of Ca^{45}. The simultaneous treatment with estrogen tended to increase the serum level of Ca^{45} (Group 6).

The present experiment supports the belief that estrogen acts independent of the parathyroids to influence calcium metabolism in bone and serum. From these observations it appears that the 2 hormones may be antagonistic in some aspects of calcium metabolism, and a proper balance is necessary for normal calcium equilibrium. If the estrogenic action

is pronounced, calcium deposition takes place in bones in greater amount and the excretion of this element is decreased. When the parathyroidal secretion predominates, calcium excretion is increased but may be retarded by estrogen. Estrogen also increases the absorption of calcium from the gut (Dallemagne *et al.*)(30), and parathyroidectomy or estrogenic treatment influences the proteins binding capacity of calcium in blood serum(31).

Summary. Administration of estrogen to normal male rats increased the radioactivity of blood serum following Ca^{45} injection, whereas bone remained unchanged. Parathyroid extract depressed radioactivity of both bone and blood serum but when estrogen was given in addition radioactivity was increased in both. Parathyroidectomy depressed the radioactivity of bones and serum but estrogen again increased the radioactivity of bones slightly and serum significantly. These data indicate that estrogen plays a role in calcium metabolism independent of the parathyroid hormone in aiding retention of calcium.

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Metabolism of Ca^{45} in Blood, Bones, and Young of Lactating Rats Treated with Estradiol.* (23088)

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In dairy cattle, with the rise in milk secretion following parturition, a condition known as "milk fever" or "parturient-paresis" may occur. In these animals both serum calcium and phosphorus are low with the result that the animal passes into a state of coma. Injection of calcium salt brings about rapid and dramatic recovery. The cause of this condition has been attributed to rapid withdrawal of blood calcium and phosphorus in synthesis of milk by the mammary gland before the parathyroid hormone secretion rate can increase to meet the greatly increased need of calcium by the mammary glands(1). In the previous study(2), the data indicated that estrogen may play a more important role in mobilizing serum calcium than had been appreciated previously. It is well known that increasing amounts of estrogen are secreted with the advance of pregnancy in dairy cattle and other mammals(3). However, following parturition and removal of the placenta, the secretion of estrogen (as shown by the excretion of estrogen in the urine and feces) is greatly reduced.

It seemed of interest, therefore, to study the calcium metabolism of normal lactating rats and compare them with similar lactating rats injected daily with an amount of estrogen estimated to equal that secreted in late pregnancy(4).

Procedure. Twelve pregnant albino rats were used. During the pre- and post-partum period, the animals were fed as previously described(2). As soon as possible after parturition (1-10 hours) the number of sucklings was reduced to 6. Six mothers were kept as controls and 6 received daily subcutaneous injections for 4 days of 1.5 μg of estradiol benzoate in 0.35 ml of olive oil. All mothers were injected intraperitoneally with total dose of 2.65 μc of Ca^{45}/100 g body weight, in the form of $CaCl_2$ (Specific activity >0.2 mc/g of Ca) over a 3 day period, starting on day of parturition. The daily gain in litter weights was recorded. Five days after parturition the mothers were killed by exsanguination from the left carotid artery. Blood and bones were treated as previously described(2). The offspring were eviscerated to eliminate variation in radioactivity that might be due to difference in amounts of milk present in the digestive system. Each newborn was digested in 5 ml hydrochloric acid. The filtrate was diluted to 25 ml with distilled water as described for bone, and 2 aliquots of each solution (1 newborn) measured for radioactivity. An effort was made to match the weights of the 2 groups of mothers. Corrections were not made for sample self-absorption. The *in vitro* addition of 17 μc of Ca^{45} to 1 ml of normal digested femur of 0.750 g (same procedure as above) gave 10,400 scintillations/minute.

Results. Average gain in weight of litters in the 2 groups was similar, indicating that estrogen did not influence secretion of milk adversely (Table I). Data for control and estrogen-treated groups show that radioactivity (Ca^{45}) in both serum and bone of the treated group was significantly higher than the controls. It would appear that in spite of intense lactation and rapid withdrawal of Ca^{45} in the milk, estrogen favorably influenced retention of Ca in the bones and in the blood serum as shown in our previous work(2). However, in the male rat the retention of Ca^{45} in bones was about twice as great as in the lactating female even though the amount of Ca^{45} injected was relatively higher. The radioactivity in blood serum in lactating animals was much lower also as compared to the male. These differences between male and lactating

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TABLE I. Effect of Estradiol upon Calcium Metabolism of Lactating Rats Injected with Ca^{45}. (Animals sacrificed on 5th day of lactation.)

	Body wt, g	Serum count, min./ml	Bone count, min./g	Gain wt, g	Radioactivity count/ min./g
		Control mothers		Litters of 6	
	255	55	7579	20.0	7737
	236	60	8608	18.0	7002
	196	48	7789	18.0	8747
	176	41	9950	22.5	6803
	166	50	7679	20.5	6639
	146	53	10192	17.0	5847
Avg	195.8	51	8632	19.3	7129
		Estrogen-treated mothers		Litters of 6	
	238	81	11839	28.0	7563
	213	84	8525	22.8	7815
	191	68	13694	18.7	5969
	166	74	19227	17.2	5639
	164	73	12660	16.6	8167
	144	72	16666	15.5	6219
Avg	186.0	75	12676	19.8	6895

female are due to the large amounts of calcium secreted in the milk.

Due to greater retention of Ca^{45} in the lactating rat treated with estrogen, it might have been expected that litters from treated groups would have shown lower radioactivity. However, the differences were slight. The lack of difference between the two groups may be due to adequacy of the calcium in milk in either group or it might be due, in part, to the presence of estrogen in milk which might favorably influence the retention of calcium in the nurslings.

Radioactivity in one femur represented 8% of total radioactivity in the body. The amount of radioactivity in the total litter was higher than that of the mother.

Discussion. The present experiment in conjunction with previous observations(2) suggests that estrogen plays a role in calcium metabolism hitherto unsuspected. Estrogen aids in the maintenance of higher levels of Ca^{45} in the blood and in lactating rats permitted greater deposition of calcium in bones in spite of great withdrawal of calcium in the milk. The mechanism of its action is not known but the observations suggest the possibilities of a higher renal or fecal threshold for calcium. Since Ca^{45} was injected intraperitoneally, effect of estrogen upon calcium absorption in the digestive tract was eliminated.

If estrogen has a similar effect upon calcium metabolism of the dairy cow, administration of estrogen post-partum to maintain the estrogen level at that time might aid in prevention of parturient paresis caused by the rapid decline in blood calcium associated with increasing milk secretion.

Summary. Estrogen, in an amount estimated to equal that secreted in late pregnancy, was injected daily into lactating rats for 4 days along with Ca^{45}. On the 5th day, radioactivity of blood serum, bones and young was compared to a similar group of lactating rats not treated with estrogen. Average gain in weight of the litters of the 2 groups was similar. However, radioactivity in serum and bones of treated group was significantly higher than in the controls. The radioactivity in litters of the 2 groups was similar. These data indicate that estrogen plays a role in the retention of calcium by the animal body.

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Paper Electrophoretic Study of Ca^{45} Binding in Sera of Normal, Parathyroidectomized and Estrogen Treated Rats.* (23089)

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Maintenance of a normal level of ionic calcium in body fluids is of great biological importance. Blood calcium exists in a diffusible and non-diffusible state, the former being biologically active and the latter bound to the plasma proteins. Equilibrium between ionized and non-ionized calcium is influenced by pH, diffusible calcium decreases with increased pH(1,2). The parathyroid hormone tends to increase the ionized calcium(3), whereas, estrogen increases the protein-bound calcium(4). These hormones may act to influence the calcium binding properties of the different plasma proteins. Visek *et al.*(5) presented evidence that Ca^{45} added to serum was readily interchangeable with the Ca^{40} present, and within a few minutes equilibrium was attained. Some investigators have used this technic to study electrophoretically the components responsible for calcium binding. Electrophoresis of cattle blood to which had been added Ca^{45} failed to show the existence of a blood fraction of higher than average specific activity(6). Clegg *et al.*(7) added Ca^{45} to the buffer solution used to dilute the serum before dialysis in an effort to increase the Ca-proteinate. In normal animals they found Ca^{45} bound to albumin; in the sera of diethylstilbestrol-treated cockerels addition of Ca^{45} reduced mobility of the leading component (albumin) which migrated to the area considered to represent the β-globulin fraction. This behavior is not clear. If addition of Ca^{45} results only in an interchange of Ca^{40} and Ca^{45}(5), and the addition of calcium scarcely increases the amount of Ca-proteinate(8), then the results of Clegg *et al.*(7) may be an artifact attributable to the procedure. This report concerns the same problem modified by *in vivo* injection of Ca^{45} prior to paper electrophoresis of blood sera of normal, parathyroidectomized and/or estrogen-treated rats.

Materials and methods. Twenty-nine adult male albino rats were divided into 4 groups and subjected to the following treatments: The control group of 8 rats received a total dosage of 2.65 μc of Ca^{45} in the form of $CaCl_2$ (specific activity$>$0.2 mc/g Ca)/100 g body weight, intraperitoneally for 4 days and sacrificed on the sixth day. The second group of 8 rats were injected subcutaneously for 7 days with estradiol benzoate, at daily dosage of 82.5 μg in 0.25 ml of sesame oil. The third group of 6 rats were parathyroidectomized (chloroform anesthesia). Group four (7 rats) were parathyroidectomized and treated similar to Group 2 with estradiol benzoate. The animals in Groups 2 to 4 received Ca^{45} during 3d to 7th days in the same dosage as controls before being sacrificed on eighth day. All animals were bled to death (chloroform anesthesia) from the left carotid artery. The sandwich technic of paper electrophoresis was employed on samples of 0.5 ml of fresh, undiluted sera using filter paper (Whatman No. 4) with a cross section of 2.5 inches and an electric field length of 11 inches. The D.C. applied was 7 milliamperes for 12 hours. Veronal solution with pH of 8.6 and molarity of 0.015 for the acid and 0.07 for the salt, was the buffer. After electrophoresis the strip was rapidly dried on horizontal rack for 30 minutes at temperature of 120° to 130°C. Seven disks 1 inch in diameter were cut in series from the paper strip for measuring radioactivity. The remainder of paper strip was stained in the normal manner (bromphenol blue) for observation of protein fractions. Sera samples were not dialyzed since the migration rates between calcium ions and protein were sufficient to separate one from the other. It was necessary to measure ra-

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dioactivity before staining because the procedure (in acid medium) dissolved the calcium from the paper strip. Radioactivity was determined by scintillation detector employing a cylindrical anthracene crystal 2.5 cm in diameter, coupled to a photomultiplier tube and decade scaler. The distance between the anthracene crystal and paper disk samples was less than 1 mm.

Results. The sandwich technic of paper electrophoresis imposes definite limitations, nevertheless it is valid to conclude from the results obtained that the ionized calcium migrated at a greater rate than the albumin. In the sera of normal animals the various protein fractions contained different amounts of Ca^{45}; the highest amounts were found in albumin and α-globulin. After parathyroidectomy there was a tendency for the amount of Ca^{45} bound to the albumin to decrease and the amount in the α-globulin to increase. In the group of estrogen-treated rats most of the Ca^{45} was in the β- and gamma-globulin components. Variations occurred in the parathyroidectomized - estrogen - treated group; highest amounts of Ca^{45} were found in either the albumin, α- or β-globulin fractions.

The present results differ from those of previous investigators. This may be due to the technic used or to species difference. Concerning the relationship between ionized and non-ionized calcium, McLean and Hastings (9) believe that Ca-proteinate behaves as a weak electrolyte and that a simple mass law equation expresses the relationship between these forms. If this is true for a single Ca-proteinate, it may not be valid for calcium binding in a complex system of proteins with different affinities and changing ratios in the sera of normal and experimental animals.

Summary. (1) In the normal rat most of the Ca^{45} is bound to albumin and α-globulin. (2) After parathyroidectomy the Ca^{45} decreases in the albumin and increases in the α-globulin fraction. (3) In animals treated with estrogen most of the Ca^{45} was present in the β and gamma-globulin components. (4) In the parathyroidectomized-estrogen-treated animals highest amounts of Ca^{45} were found in either albumin, α- or β-globulin fractions.

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Sensitivity to Analogs of Thiamine Pyrimidine Associated with Resistance To Amethopterin or Purine Antagonists.*† (23090)

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Law(1) reported that certain lines of leukemia in mice became more sensitive to amethopterin when resistance to 6-mercaptopurine (6-MP) developed, or when resistant to, or "dependent" upon, 8-azaguanine (8-AG). Szybalski and Bryson(2) observed an instance of mutation in *Escherichia coli* wherein increased resistance to one antibiotic was associated with increased sensitivity to a second. They designated this phenomenon "collateral sensitivity." Subsequently, Elion, *et al.*(3) found a 2-6-diaminopurine-resistant strain of *Lactobacillus casei* that possessed increased sensitivity to 6-MP. Elion and Hitchings reported a 6-MP-resistant strain of *L. casei* with increased sensitivity to amethopterin, and conversely Hutchison and Burchenal(4) observed collateral sensitivity to 6-MP in an amethopterin-resistant *Streptococcus fecalis* strain. Schabel, Wheeler and Skipper reported that an azaserine-resistant strain of *E. coli* was more sensitive than the parent to 6-MP and diaminobiuret, and that a sulfanilamide-resistant strain showed collateral sensitivity to 6-chloropurine, 8-AG or purine.

The phenomena of collateral sensitivity and cross resistance have been used in this laboratory to screen compounds of potential interest in cancer chemotherapy. Since amethopterin and 6-MP are of interest in clinical acute leukemia, and resistance to them apparently arises in leukemic cells of rodents and humans, mutant organisms resistant to these two antimetabolites served as a "collateral sensitivity screen."

Materials and methods. Several amethopterin-resistant mutants were isolated from populations of the parent, *Bacillus subtilis* ATCC 6051, and from a purineless auxotroph, *B. subtilis* 6051-9. Mutants resistant to 6-MP were isolated from a purine-requiring *E. coli* strain, 9661-01 (P-)(5). From cultures of the 6-MP-resistant organisms, 2 mutant strains were isolated that were resistant simultaneously to 8-AG, 6-thioguanine (6-TG) and 6-MP. In all cases, drug-resistant mutants were isolated from solid agar media by incorporating large populations of the sensitive parent into minimal agar medium containing the drug, incubating, and sub-culturing discrete colonies that appeared. Using these resistant strains, 245 compounds were tested, including 175 pyrimidines, 22 purines, 5 sulfonamides, 2 antibiotics, 24 naturally occurring amino acids, azaserine and 16 miscellaneous compounds. Each compound was tested alone and also in combination with amethopterin or 6-MP, to detect possible synergistic effects. Any compound that produced growth-inhibition upon initial screening against resistant mutants was tested again upon the parent organism and the resistant mutant, under identical conditions, to compare sensitivity of parent and mutant. If the amethopterin- or 6-MP-resistant mutant consistently showed greater sensitivity to the test compound than the parent in 3 or more replicate experiments, the resistant strain was considered to possess collateral sensitivity to the test compound. The agar-diffusion method was used for routine testing. Two different minimal, chemically-defined agar media, one for each bacterial species(5) were homogeneously seeded with the test organism and distributed in Petri dishes. Each compound was tested by impregnating filter paper discs (12.7 mm diameter) with solutions containing approximately 0.5 μM and placing them upon the surface of the agar. The width of inhibitory zone, if any, as well as completeness of inhibition within the zone, was noted. In growth experiments with *B. sub-*

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TABLE I. Collateral Sensitivity to Amethopterin and to Certain Analogs of Thiamine Pyrimidine Exhibited by an *Escherichia coli* Mutant Resistant to 3 Purine Antagonists.* No inhibition was found in parent strain.

	Compound tested†		mm diam. inhibition zone
	R‡	X‡	Mutant
1.	HS–	–OH	0
2.	CH_3S–	–OH	34
3.	CH_3CH_2S–	–OH	0
4.	CH_3S–	$-OCH_2CH_3$	30
5.	CH_3CH_2S–	–Cl	34
6.	HO–	$-NH_2$	34
7.	Amethopterin		36

* 6-mercaptopurine, 8-azaguanine, 6-thioguanine.
† 0.05 cc of M/100 sol. impregnated in 12.7 mm paper disc, placed upon agar surface.
‡ 2-R-4-amino-5-Xmethyl pyrimidine.

tilis in liquid medium, agar was omitted but otherwise the same minimal medium was used. The medium was distributed in 6 cc amounts in culture tubes of convenient size (18 x 150 mm) for use in determination of optical density. These tubes were placed in a slanted position upon the platform of a rotary shaker. These conditions had been previously demonstrated to give adequate aerobiosis.

Results. In the course of testing growth inhibitory effects of 239 compounds against resistant mutants, only one compound, amethopterin, produced greater inhibition of a drug-resistant mutant than for the parent (collateral sensitivity). Two *E. coli* mutants, resistant to purine-antagonists, were more sensitive than the parent strain to amethopterin. This is shown for one mutant in Table I. Subsequently, a second case of collateral sensitivity was demonstrated for amethopterin-resistant mutants of *B. subtilis* in the following manner. It was observed that 2-methyl - 4-amino - 5-aminomethyl pyrimidine (2-CH_3-AAMP), the pyrimidine moiety of thiamine, inhibited *B. subtilis* 6051-9/A-1, a purine-requiring, amethopterin-resistant, double-mutant. When the medium was supplemented with amethopterin, however, the inhibitory effect was prevented. Because of this unexpected finding, the effect of 2-CH_3-AAMP upon amethopterin-inhibition of the wild-type parent was tested. This pyrimidine was found to block amethopterin-inhibition in this organism completely. Subsequently, studies of thiamine and cocarboxylase revealed these metabolites to be effective in preventing amethopterin-inhibition.

Because of these results, 6 analogs of the thiamine pyrimidine were obtained‡ and tested. All of these produced growth-inhibition of various strains of *B. subtilis* or *E. coli* that could be specifically blocked by thiamine pyrimidine. Results of attempts to demonstrate collateral sensitivity with these compounds are shown in Tables I and II. Three analogs of the pyrimidine moiety of thiamine, compounds 2, 4 and 5 in Table I, were the only compounds for which significant collateral sensitivity was found for both *B. subtilis* strains resistant to amethopterin, and for an *E. coli* strain resistant to certain purine antagonists. Compound 1 and 3 elicited collateral sensitivity in the *B. subtilis* strains only. Collateral sensitivity to compound 2 in two amethopterin-resistant *B. subtilis* mu-

TABLE II. Collateral Sensitivity to 2-CH_3S-AHMP* in 2 Amethopterin-Resistant *B. subtilis* Mutants.

Strain	Supplement	Optical density‡	% inhibition
6051 (parent)		.49	
	Ameth.†	.07	87
	2-CH_3S-AHMP*	.39	18
6051/A-2		.67	
	Ameth.	.68	0
	2-CH_3S-AHMP	.19	70
6051/A-3		.64	
	Ameth.	.63	3.8
	2-CH_3S-AHMP	.23	63

* 2-methylmercapto-4-amino-5-hydroxymethyl pyrimidine, 10^{-5} M.
† Amethopterin, 10^{-6} M.
‡ Optical density of culture medium. Avg four consecutive experiments. Growth 16 hr on rotary shaker at 25°C.

‡ Thiamine pyrimidine analogs were obtained through kindness of following workers: (1) 2-methylmercapto-4-amino-5-hydroxymethyl pyrimidine, from Dr. J. Gots. Prepared by Ulbricht and Price(6); (2) 2-mercapto-4-amino-5-hydroxymethyl pyrimidine, and (3) 2-ethylmercapto-4-amino-5-hydroxymethyl pyrimidine, from Dr. C. C. Stock; (4) 2-methylmercapto-4-amino-5-ethoxymethyl pyrimidine, Dr. J. Hoover; (5) 2-ethylmercapto-4-amino-5-chloromethyl pyrimidine, and (6) 2-hydroxy-4-amino-5-aminomethyl pyrimidine, from Dr. J. M. Sprague.

tants is demonstrated in Table II. One of the 6 pyrimidines, 2-hydroxy-4-amino-5-aminomethyl pyrimidine (2-HO-AAMP), differed from the others. Although growth of parent strain of *B. subtilis* was inhibited by this compound, none of the amethopterin-resistant mutants were sensitive to it, but instead showed cross-resistance, the converse of collateral sensitivity. Yet, the *E. coli* mutant resistant to 3 purine antagonists demonstrated collateral sensitivity to 2-HO-AAMP (Table I). These results indicate that one cannot predict from the effects of a pair of antimetabolites in one biological system, whether cross-resistance or collateral sensitivity will be demonstrated in a second system.

It is noteworthy that the only other compound for which *E. coli* strains resistant to purine antagonists exhibited collateral sensitivity was amethopterin, an antimetabolite of demonstrated value in acute leukemia. The results reported here support the view that, at least in some cases, collateral sensitivity to a second drug accompanying resistance to a first drug, has its basis in rather close metabolic relationships between metabolic targets of the two drugs. In this respect, the phenomenon is closely related to the inverse phenomenon of cross-resistance. The discovery of collateral sensitivity in both 6-MP and amethopterin-resistant mutant organisms for several members of a new series of pyrimidines, all analogs of the pyrimidine moiety of thiamine, permits new experiments in two directions. Work is in progress to assess the value of these pyrimidines in animal neoplasms where the effects of 6-MP and amethopterin are known, and where resistance to these agents has been established. One analog, 2-CH_3S-AHMP, in preliminary experiments has been found to retard significantly leukemia and carcinoma in mice. Elucidation of biochemical pathways relating the folic acid complex to thiamine synthesis may follow from studies of the mode of action of amethopterin and of the thiamine pyrimidine analogs in *B. subtilis* and *E. coli*. Such studies have been initiated in this laboratory.

Summary. A microbiological method for biochemical screening of compounds of potential interest in the chemotherapy of leukemia, using mutants of *B. subtilis* resistant to amethopterin and *E. coli* mutants resistant to purine-antagonists, has been described. Greater growth suppression by a test compound in the resistant strain, when contrasted to the parent strain, has been sought (collateral sensitivity). This phenomenon of collateral sensitivity has been observed for several chemical analogs of the pyrimidine moiety of thiamine. Possible significance of these findings for studies in intermediary metabolism and for cancer chemotherapy has been discussed.

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Cultivation in Tissue-Culture of Cytopathogenic Agent from Bovine Mucosal Disease.* (23091)

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Since 1951 bovine mucosal disease has been reported from 20 states and Canada. Because the symptoms of mucosal disease are similar to those of other virus diseases it is believed that many cases are not diagnosed. The disease is characterized by ulceration of mucous membranes of oral cavity and of the digestive tract, increased temperature, nasal and lachrymal discharges, and diarrhea. Mucosal disease as seen in field cases has a characteristic morbidity of 2-10% with case mortality of 95%. The etiology of the disease is unknown. Ramsey(1) and Pritchard(2) showed that transmission studies made in cattle using macerated tissues and whole blood were inconclusive. Preliminary studies here presented suggest the difficulty in finding experimental animals without some degree of immunity.

To date nothing has been reported in the literature on tissue-culture isolations of agents from cattle with mucosal disease. This is a report of 2 isolations from material obtained from tissues from cases of bovine mucosal disease and a study of some characteristics of these viruses.

Materials and methods. Bovine kidney cells used for tissue-cultures were obtained from fetuses 12-15 inches long. Kidney tissues were trypsinized and prepared according to the method of Young *et al.*(3). The cells were started on medium containing 20% adult bovine serum (BoA) and 0.5% lactalbumin hydrolysate (LA) in Hanks'(6)(H) balanced salt solution. Penicillin (100 units) and streptomycin (100 μg)/ml were added to all basal media. Tissues were cultivated in Pyrex tubes (12 x 75 mm) each containing a coverslip (Corning 8 x 22 mm), and sealed with No. 00 silicone stopper. The tubes were seeded with approximately one million kidney cells in one ml of medium (BoA 20% + LA + H), placed in horizontal rack at 12° angle, and incubated at 37°C. Tissue cultures were inoculated with the virus material after incubating one week. To remove any antibodies present from the adult bovine serum used for initial growth, the tissue cultures were washed with medium (BoN 5% + LA + H) prepared with antibody-free serum. This serum was obtained from a newborn calf which had never nursed. After the second wash the medium was removed and replaced with one ml of BoN 5% + LA + H in which the appropriate dilution of virus was suspended. The cultures were again incubated at 12° angle and 37°C. *Virus materials* were obtained from 2 sources: M-833, a pool of tissues from a mucosal disease outbreak in central Nebraska herd(4) and ISC-1, a pool of lymph nodes obtained from animal with mucosal disease from Iowa herd(5). In preparation for inoculation, tissues were minced with washed ground glass (Pyrex) in mortar and diluted 1:10 with BoN 5% + LA + H. This material was then centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was then withdrawn and treated with 1000 units of penicillin and 1000 μg streptomycin/ml for one hour at 4°C. Following this treatment a final dilution of 1:100 with BoN 5% + LA + H was prepared for inoculating the washed tissue cultures. In preparation for transfer of the virus from the tissue cultures (5th-7th day), approximately 0.5 g of sterile ground glass was added to each tube. The cultures were then shaken for 10 minutes to disrupt the cells. The supernatant was removed with capillary pipette, treated with 1000 units of penicillin and 1000 μg streptomycin, and centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant ma-

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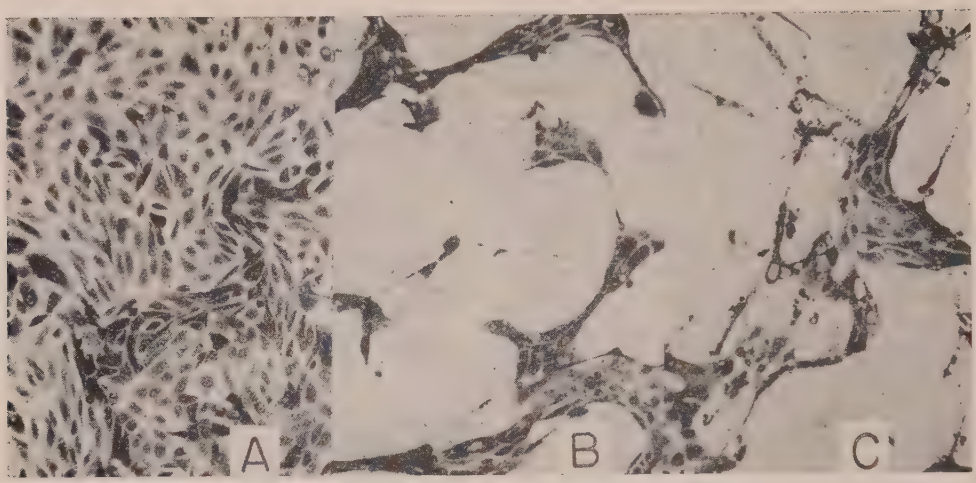

FIG. 1. Photomicrographs of May-Grünwald-Giemsa stained bovine kidney tissues. (A) Normal tissue 12 day control, (B) tissue 12 days following inoculation with virus strain M-833, (C) tissue 13 days following inoculation with virus strain ISC-1.

terial was either quick frozen and stored at −20°C or diluted 1:100 with BoN 5% + LA + H for reinoculation into washed tissue cultures. The cultures were examined microscopically for cytopathogenic changes by viewing living cells on tube wall and coverslip. When the cell changes were noted the coverslip was removed aseptically with the split end of a sterile applicator stick, stained by the May-Grünwald-Giemsa method(6), and permanently mounted on slide.

Results. Following 7-10 days incubation of virus and tissue, cytopathogenic changes were apparent. These changes started with small vacuoles forming within the cells and increasing in size until the cell disintegrated on about 10th-14th day. Fig. 1 shows photomicrographs of May-Grünwald-Giemsa stained tissues. Identity is as follows: (A) Normal tissue control stained 12 days after washing and feeding with BoN 5% + LA + H. This layer, composed mainly of epithelial cells, covered entire coverslip and was typical of growth obtained with bovine embryonic kidney cells; (B) and (C) Bovine kidney cells inoculated with virus strains M-833 and ISC-1. Both of these virus strains have the same general pattern of attack. These typical photographs show large areas of cell destruction which is followed by further disintegration until all of the tissue is completely destroyed.

Preliminary studies with virus strain M-833 show that it has an 11th passage titer of 10^{-5} (total accumulated dilution titer 10^{-22}), is inactivated over 50°C for 15 minutes, and will pass 03 Selas filter. Serial passages in an attempt to adapt the virus to 7-day embryonated chicken eggs by yolk sac inoculations were negative. The virus strain ISC-1 was also inactivated by temperatures over 50°C for 15 minutes and passed an 03 Selas filter. During primary isolation of strain M-833, 3 unrelated viruses and a tissue control were also serially passed in kidney tissue cultures from the same fetus. None of these developed cytopathogenic changes, alleviating the possibility that the agent was isolated from the kidney tissue. Strain ISC-1 was isolated later using kidney cells from a different fetus.

Serological studies were done in sheep with virus strain M-833 sixth passage material. Two sheep were each given one ml of the virus tissue culture supernatant material intravenously at weekly intervals for 3 weeks. The sheep were bled prior to initial inoculation with virus and 3 weeks following final inoculation. The results are presented in Table I and indicate that virus strain M-833 stimulated formation of neutralizing antibodies. In cross-neutralizing tests the antibodies stimulated by M-833 were also capable of neutralizing virus strain ISC-1. This suggests a relationship between the 2 strains.

TABLE I. Neutralization of M-833 and ISC-1 Viruses by Serum from Sheep Immunized with M-833 Virus.

	Virus M-833		Virus ISC-1	
	Pre-inoculation*	Convalescent*	Pre-inoculation*	Convalescent*
Sheep 145	—	+	—	+
160	—	+	—	+

* All serums diluted 1:10.
— = Antibody absent; + = Antibody in sufficient amounts to neutralize approximately 1000 infective doses of virus.

A preliminary study of bovine serum samples from 6 widely separated herds indicates that antibodies capable of neutralizing this agent are common in herds having no history or symptoms of mucosal disease. Formation of these neutralizing antibodies resulted from an infection of unknown etiology. Neutralizing antibodies were generally not found in serum samples from younger range cattle.

Although this viral agent was isolated from lesions of cattle having mucosal disease, the evidence presented does not prove it to be the causative agent of this disease. This proof can only be obtained by establishing the disease with this virus in a susceptible calf. The presence of neutralizing antibodies in the calves available for experimentation makes it impossible to conduct a study of this type at the present time. Experimental work along these lines would, of necessity, require disease-free, antibody-devoid calves taken by caesarotomy or hysterectomy and raised in strict isolation or preferable in Horsfall-Bauer type units designed for calves. These experimental studies will be undertaken as soon as these conditions can be provided.

Summary. Isolation and cultivation in tissue culture of a cytopathogenic agent recovered from tissues of cattle with mucosal disease from 2 separated herds is reported. A homologous tissue culture system was used with bovine embryonic kidney cells and newborn, antibody-free calf serum. The virus was inactivated by temperatures above 50°C for 15 minutes, would pass an 03 Selas filter and had an 11th passage titer of 10^{-5}. In sheep, the virus stimulated formation of neutralizing antibodies. A preliminary neutralization study indicated that antibodies are common in animals from herds having no previous history of mucosal disease.

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Studies on Deoxyribosidic Growth Requirement of *Lactobacillus acidophilus* R-26. (23092)

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(Introduced by A. J. Dalton)

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Recent independent studies of acid soluble extracts of liver(1,2) and thymus(3) have demonstrated the presence of different types of deoxyribose compounds in these tissues. In liver, the nucleoside, deoxycytidine, was isolated and shown to account for an average of 92% of the total deoxyribosidic compounds present, while 5′ mono-, di-, and triphosphates of deoxycytidine and thymidine were isolated from thymus. The relative proportions of the latter compounds have not been established. Since in the study of liver, deoxyribosidic compounds were determined by growth of *L. acidophilus* R-26, the results were contingent upon specificity of the deoxynucleosidic requirement of this organism. To resolve these differences, the ability of the organism to utilize di- and triphosphates of pyrimidine deoxynucleosides was tested. The results indicate that the organism was unable to grow in the presence of these compounds.

Materials and methods.† Ability of various deoxyribose containing compounds to support growth of the organism, *L. acidophilus* R-26, was determined essentially as described previously(1,4) using a modified medium suggested by Dr. E. Hoff-Jorgensen (personal communication). Pure thymidine served as standard growth supplement for this organism. Ability of other compounds to support growth was expressed in terms of micromoles of thymidine. The highest concentration at which compounds with low activity were tested, however, was usually limited to 100 times the concentration of pure thymidine required to produce growth. Compounds tested for activity included the following: commercial calcium TMP‡ and barium 3′, 5′-TDP,‡ TDP, TTP, D-CDP, and D-CTP isolated from thymus(2) or synthesized(5) using modifications of the method of Hall and Khorana(6), and 3 samples of TMP isolated from enzymic or acid hydrolysates of TDP or TTP. Concentration of these compounds was calculated from absorption of neutral solutions at 260 mμ using the constant 8400 for thymidine containing solutions and 7400 for deoxycytidine containing solutions(7). Deoxyribose content of solutions was determined by the cysteine-sulfuric acid reaction(8). In our experience, the results with this method can vary as much as ±

TABLE I. Deoxyribose Content and Microbiological Activity of Deoxynucleotides.

Compound	Method of sterilization*	Deoxyribose content†	Microbiological activity‡ Before hydrolysis	Microbiological activity‡ After hydrolysis§
TTP	A	.81	.13	.69
"	F		.011	.69
D-CTP	F	.86	<.005	.95
D-CDP	F	.87	.052	.84
TDP	F	.78	.48	.67
3′, 5′-TDP	A		.14	
"	F		.055	
TMP‖	A		1.02	
" ¶	A	.88	.94	
" **	A	1.02	.88	
" ††	A	.68	.83	

* A = autoclaving; F = filtration.
† Micromoles/micromole nucleotide.
‡ Expressed as micromoles thymidine/micromole nucleotide.
§ Hydrolysis was for 10 min. at 100° in 1 N HCl.
‖ Commercial sample; 100 ± 3% pure.
¶ Isolated from apyrase hydrolysate of thymus TTP.
** Isolated from apyrase hydrolysate of thymus TDP.
†† Isolated from acid soluble fraction of thymus.

* Supported by Atomic Energy Commission contract.

† The following abbreviations will be used: TMP, TDP, and TTP, the 5′ mono-, di-, and triphosphates of thymidine; 3′,5′-TDP, thymidine 3′,5′ diphosphate; D-CDP and D-CTP, the 5′ di- and triphosphates of deoxycytidine; DNA, deoxyribonucleic acid.

‡ Obtained from California Foundation for Biochemical Research, Inc.

TABLE II. Deoxyribose and Phosphate Content and Microbiological Activity of Natural and Synthetic Deoxynucleotides.

Compound*	Deoxyribose content†	After sterilization by filtration		After hydrolysis in 1 N HCl	
		Thymidine‡	Inorganic phosphorus§	Thymidine‡	Inorganic phosphorus§
TDP-I	.93	.11	.14	.81	.99
" -S	.96	.04	.03	.79	.96
D-CDP-I	.84	.15	.17	.77	.89
" -S	1.01	.02	.03	.81	.88
TTP-I	.98	<.01	.061	.79	1.95
" -S	.97	"	.099	.83	1.98
D-CTP-I	.80	"	.088	.82	1.77
" -S	1.00	"	.079	.94	1.90
TMP		.95		.77	

* I = isolated from thymus; S = synthetic. † See Table I. ‡ Micromoles/micromole nucleotide as measured microbiologically. § Micromoles/micromole nucleotide as measured by method of Soyenkoff(9).

10%. Phosphorus was determined colorimetrically by the method of Soyenkoff(9).

Results. Hoff-Jorgensen(4) demonstrated previously that the organism *L. acidophilus* R-26 would produce the same amount of growth when supplemented with equimolar amounts of any of the 5 known deoxynucleosides. On the basis of an experiment in which amount of growth obtained with a deoxyribonuclease digest of DNA was the same as its deoxynucleotide content, he also concluded that deoxynucleotides were utilized to the same extent by the organism as deoxynucleosides. Although the correctness of this conclusion was placed in doubt by the experiments of Sinsheimer(10) which showed that deoxyribonuclease digests of DNA contained only 1% of their nucleotide content as simple mononucleotides, any of the pure natural deoxynucleotides gave the same growth of the organism on a molar basis as the deoxynucleosides(1) (see also Tables I and II).§

In contrast to the ability of deoxynucleosides and deoxynucleotides to permit growth of *L. acidophilus* R-26, a sample of TTP isolated from thymus permitted only 13% as much growth as thymidine on the basis of nucleotide content (Table I). Since the compound had been autoclaved with the medium in this experiment, and since triphosphate solutions are known to be unstable at elevated temperatures, the TTP solution was sterilized by filtration‖ and added to the autoclaved medium. Under these conditions, the compound exhibited only 1.1% of the activity anticipated (Table I). A solution of sample of D-CTP had even less activity while D-CDP and commercial 3′, 5′-TDP had about 5% of the activity expected from their nucleotide content when sterilized by filtration. On the other hand, a sample of TDP isolated from thymus had 48% of the growth activity expected after sterilization by filtration. The latter result was entirely unexpected since the other 4 di- or triphosphates gave much lower activity when tested under these conditions, and it was suspected that the thymus TDP had undergone extensive breakdown before it was tested.

To determine whether phosphorylated deoxynucleotides isolated from thymus had the potential to promote growth of *L. acidophilus* R-26, they were hydrolyzed in 1 N HCl to remove the labile phosphate groups. After this treatment, the compounds gave 67-95% of the activity expected from their nucleotide content (Table I). These values were in rough agreement with the deoxyribose content determined colorimetrically (Table I).

§ In preliminary experiments with Dr. R. L. Sinsheimer, 2 dinucleotides isolated from a deoxyribonuclease digest of DNA gave the same growth as if the nucleotides they contained were completely utilized. These results and also results of tests of other polynucleotide components of deoxyribonuclease digests of DNA will be reported later.

‖ Sterilization was carried out with ultra fine fritted glass filter (Corning No. 33992).

Although results of the experiments reported in Table I strongly suggested that deoxynucleoside di- and triphosphates were unable to support growth of the organism, the high activity observed with the TDP sample and the slight activity of the other 2 diphosphates left some doubt as to whether these compounds might possess some intrinsic ability to promote growth of the organism. In a second series, new samples of deoxynucleoside di- and triphosphates were tested. These included samples purified from thymus extracts as well as synthetic preparations. The results, Table II, show that diphosphates isolated from thymus had activities of 11 and 15% while corresponding synthetic samples had activities of only 4 and 2%, respectively. Both natural and synthetic samples of the deoxynucleoside triphosphates were inactive when tested at concentrations sufficient to detect activity at the 1% level.

The variable activities of the deoxynucleoside diphosphates (Tables I and II) could best be explained on the assumption that the different samples had decomposed in varying degree to inorganic phosphate and the corresponding deoxynucleotides, which would, of course, be fully utilized by the organism. That this was the case was strongly indicated by the close correlation between the inorganic phosphate content of the diphosphate samples and their ability to promote growth of the organism (Table II). Decomposition would be expected to be a less serious problem with the triphosphate samples, as far as growth promoting activity was concerned, since 2 phosphate groups would have to be lost before microbiological activity could occur. The fact that the triphosphates (Table II) had less than 1% of the anticipated activity although their inorganic phosphate contents varied from 6.1 to 9.9% indicated that decomposition had proceeded only to the diphosphate stage.

After hydrolysis in acid to remove the labile phosphate groups, the natural and synthetic phosphorylated deoxynucleotide samples exhibited high activity, ranging from 77-94% of that expected from their nucleotide contents (Table II). In this series of experiments the effect of acid hydrolysis on microbiological activity of TMP was also tested. A 20% reduction in the ability of this compound to support growth occurred. This loss in activity may account in part for the discrepancies between microbiological activities of the hydrolyzed compounds and their deoxyribose contents.

Discussion. Although our results clearly show that pyrimidine deoxynucleoside polyphosphates cannot satisfy the deoxyribosidic growth requirement of the organism, *L. acidophilus* R-26, it should be possible to convert these compounds enzymatically to deoxynucleosides or deoxynucleotides so that the organism could be used to determine these polyphosphates in biological material.

Additional experiments will be required to determine whether failure of the organism to utilize deoxynucleoside polyphosphates was due to impermeability of the bacterial cell wall or to inability of the organism to convert the compounds to a usable form.

Summary. Our experiments indicate that *L. acidophilus* R-26 could not use either 3′, 5′-TDP, TDP, TTP, D-CDP, or D-CTP for growth, although the compounds were fully utilized after hydrolysis. It was concluded that the organism could not be used directly to detect deoxynucleoside polyphosphates in tissue extracts.

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Heparin in Blood.* (23093)

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The problem as to whether heparin is present in blood as a normal constituent has been investigated by a number of workers(1-9). The results of these studies are conflicting because even if heparin is present in blood, its concentration is very small. Isolation of heparin from normal blood and unequivocal identification could consequently not be accomplished by ordinary means. In a recent report(10), we have shown that exogenous inorganic sulfate can act as a precursor for the sulfate ester groups of heparin. Thus, when sodium sulfate containing S-35 is injected into dogs, the heparin which is subsequently isolated from the liver is radioactive. On the basis of this finding, it was expected that if heparin is a normal constituent of blood it should be susceptible to identification on the basis of its radioactivity in animals which had previously been treated with radioactive sulfate. The present report describes the procedures and the results obtained.

Methods. Dogs weighing 16 to 19 kg were injected intraperitoneally with 12 ml of an aqueous solution of $Na_2S^{35}O_4$ (10 mg) containing 1.3 x 10^9 c.p.m. and blood was drawn from different animals after 26 and 48 hours. Subsequent to addition of sodium heparin (Lederle) as a carrier, 100 ml of blood were extracted with 100 ml 0.5 N NaOH and 14 ml saturated ammonium sulfate at 50° for 1.5 hours. The solution was then heated to 75°, centrifuged and washed twice with 50 ml 0.06 N NaOH and 5 ml saturated ammonium sulfate. The supernatant and washings were combined and concentrated at room temperature to 50 cc. Acidification with H_2SO_4 to pH 2 yielded a precipitate which was collected, washed with dilute H_2SO_4, extracted with ethanol and dried. The subsequent steps involving digestion with trypsin and reprecipitation from different solvents were similar to those described for isolation of labeled heparin from liver(10). The barium acid salt which was finally obtained was recrystallized 3 times and similar values for radioactivity were obtained after each recrystallization. Eighteen ml blood samples were also extracted by the procedure of Monkhouse and Jacques(8) subsequent to addition of 100 mg of carrier heparin. The aqueous solution, after extraction with phenol, was dialyzed, passed through Dowex 2 anion exchange resin, purified and recrystallized in a manner similar to the first procedure. Radioactivity measurements were made on 1.2 to 1.6 mg aliquots with a thin window gas flow counter on plates having an area of 8 sq. cm. It was found that these conditions gave infinitely thin samples so that no absorption corrections were necessary.

TABLE I. Radioactivity of Heparin Isolated after Addition of Carrier.

Time (hr)	Recrystallization	Total count	mg recovered	Cpm/100 mg
26	1	2475	45.9	5392
	2	2068	38.8	5381
	3	2020	37.6	5372
48	1	2205	48.6	4537
	2	1903	42.0	4531
	3	1359	30.0	4530

Results. Table I shows the results obtained by the first method. It can be seen that a significant amount of radioactivity can be detected in the heparin which is isolated from blood. If labeled inorganic sulfate had been present in the original blood, it was completely eliminated in the process of isolation and purification. That this is so has been shown in studies with liver(10) and in control experiments with blood to which $Na_2S^{35}O_4$ and heparin were added.

Recrystallization of the barium acid salt did not reduce specific activity appreciably (Table I), further indicating the radioactive purity of the heparin. The material had an anticoagulant activity of over 100 U.S.P. units per milligram and was inhibited by pro-

* A portion of this report was presented before meeting of A.A.A.S. in New York, Dec. 26, 1956.

tamine as would be expected for heparin. Analysis showed it to contain 23.52% barium and 19.2% glucosamine. Radioactivity of the blood heparin 48 hrs. after administration of the radioactive sulfate is less than 24 hours. This has also been observed when heparin was isolated from liver. The reason for this is that the time of maximum incorporation of the label is about 30 hours after sulfate injection.

Extraction of blood heparin by the second procedure gave material of much lower specific activity. Furthermore, the amount of radioactivity obtained could not be duplicated from one experiment to the other. Data obtained by this method were, therefore, not considered sufficiently reliable for tabulation.

Discussion. The activity due to radioactive heparin in 100 ml blood would be in the order of 5300 c.p.m., 26 hours subsequent to administration of the radioactive heparin. The liver heparin isolated at this time has a specific activity of 10^4 c.p.m./mg.

Since maximum incorporation into liver heparin is not reached until after 26 hours, it would be expected that its activity in the blood would be lower at this time, assuming that heparin is formed in the liver. The conclusion might consequently be made that normal blood contains at least 0.5 mg of heparin/100 ml.

The second extraction procedure (*i.e.* Monkhouse & Jacques(8)) yielded heparin with less than half the specific activity obtained by the first method. It would, consequently, appear that the phenol procedure does not completely extract heparin from the complex in which it exists in the blood. To release heparin from this complex, digestion with proteolytic enzymes seems to be essential. This may also be the reason why Charles and Scott(3) and the others who have used their method found heparin in blood, while Monkhouse and Jacques(8) did not. Further studies to verify this conclusion are being conducted.

Summary. The presence of heparin in normal blood is demonstrated by the "carrier" technic, using radioactive sulfate as precursor. It appears to be found in blood in a combined form so that preliminary decomposition of the complex is required before it can be isolated.

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Use of Urea and Salicylate to Elute Antibody from Insoluble Antigen.* (23094)

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As a part of research directed toward the use of antibodies as carriers of radioactivity for therapeutic purposes we are investigating technics of dissociating and rendering soluble an antibody bound to insoluble antigen. Methods commonly used have involved either acid ≈ pH 3.2(1), alkali ≈ pH 11(2), or heat, 60°C(3). Our recent experiments show that some hydrogen bond forming reagents, in particular urea and sodium salicylate, can also be used effectively and conveniently for this purpose. At concentrations breaking antigen-antibody bonds no denaturation or other alteration of rabbit gamma globulin has been detected.

Experimental. Elution with urea. By general methods previously described(4,5) a partially purified preparation of I^{131} labeled rabbit anti-rat Walker carcinoma 256 antibodies was obtained in which approximately 30% of the I^{131} was attached to antibodies that would bind to tumor residue on *in vitro* incubation. The test system used was to incubate with shaking 0.5 ml portions of this anti-tumor preparation with 1 ml of tumor homogenate plus buffer and normal rabbit serum for 1 hour at 37.5°C, using technics previously described(4,5). The residues obtained after centrifugation were washed with pH 8 borate buffer and the bound antibody eluted with urea under varied conditions indicated in columns 2-6 of Table I. Column 7 shows the per cent elution measured as I^{131} remaining in solution in the supernatant after centrifuging. To test specificity of the eluted labeled antibody for rat tumor compared with rabbit tissue, portions of these eluates were incubated once more with rat tumor and with rabbit kidney homogenates. By dilution the urea concentration was below 2.3%. Fixation of labeled antibody to the washed residues is shown in columns 8 and 9. For comparison, the binding to rat tumor and rabbit kidney homogenates of the I^{131} antibody preparation before absorption onto tumor and urea elution is shown as the last 3 experiments in this table. Comparison with the earlier values indicates that all conditions tested for urea elution gave a product with greater specific uptake by rat tumor and less nonspecific uptake by rabbit kidney than found for the untreated preparation. However, increased temperature and higher pH, as well as higher urea concentrations, within the range of the experiment were associated with larger yields of eluted labeled antibody. Further experiments indicated that overnight storage in 16% urea at 2-3°C did not adversely affect eluted antibody as measured in this type of experiment.

Elution with sodium salicylate. Similar experiments were made using solutions of sodium salicylate as an eluting medium, with similar results except that salicylate was active at somewhat lower concentrations; 70% of tissue-bound antibody could be solubilized by stirring it in a 10% sodium salicylate solution for 5 minutes at room temperature. The salicylate eluted antibody reacted similarly to the urea eluted antibody when tested with rat tumor and rabbit kidney homogenate, and seemed not to be harmed by overnight storage at 3-4°C in 10% salicylate solution.

In vivo degradation studies of urea and salicylate treated rabbit gamma globulin. McFarlane has presented convincing evidence (6) that a very sensitive indication of non-physiological alteration or denaturation of blood plasma proteins is an increased rate of elimination from the blood compared with similar unaltered proteins. Twenty mg of gamma globulin were therefore obtained by ammonium sulfate fractionation of normal

* This paper is based on work performed under contract with U.S.A.E.C. at University of Rochester Atomic Energy Project, Rochester.

TABLE I. Description of Conditions of Urea Elution of I^{131} Labeled Anti-Walker Tumor Antibodies, % Elution, and Uptake of Eluted Antibody by Tissue Homogenates.

% urea	Solvent	Vol urea, ml	Temp., °C	Elution time, min.	% elution	Uptake by Tumor homog.	Uptake by Rabbit K homog.
16	Dist. H_2O	4	23	15	56.4	50.1	5.6
32	*Idem*	"	"	"	72.2	49.6	6.4
16	"	"	"	5	51.9	49.0	5.7
"	"	"	"	"	54.1	47.9	4.5
"	"	"	37.5	"	59.8	46.3	5.9
"	"	2	23	"	41.3	47.7	4.8
"	"	6	"	"	57.6	48.8	6.3
32	"	4	"	"	71.2	47.2	5.6
16	pH 8 PO_4 buffer	"	"	"	41.5	44.4	8.4
"	9 " "	"	"	"	54.1	47.9	8.2
"	10 " "	"	"	"	73.0	48.2	6.6
"	5 c a* "	"	"	"	49.6	58.4	4.9
"	4 " "	"	"	"	37.8	58.3	2.9
32	Dist. H_2O	"	3–4	"	47.7	58.5	6.4
24	*Idem*	"	23	"	65.6	53.9	5.6
8	"	"	3–4	"	13.1		
"	"	"	23	"	19.3		
—	—	—	—	—	—	30.3	13.8
—	—	—	—	—	—	30.8	14.3
—	—	—	—	—	—	30.5	13.9

* c a = citric acid.

rabbit serum, trace labeled with I^{131}, and divided into 3 portions. One portion was diluted with an equal volume of saline, one with an equal volume of 20% sodium salicylate, and one with 64% urea. After stirring 5 minutes at room temperature the solutions were centrifuged and the supernatants dialyzed for 17 hours against saline. After centrifuging, portions of each solution were injected intravenously into 2 rabbits and 2 rats. Fig. 1 shows the relative radioactivity of blood taken from the 6 rabbits, with the first blood samples, taken 1.5 hours after injection, given a normalized value of 100. Fig. 2 shows the relative radioactivity of the injected rats, as measured in terms of an I^{131} standard with an

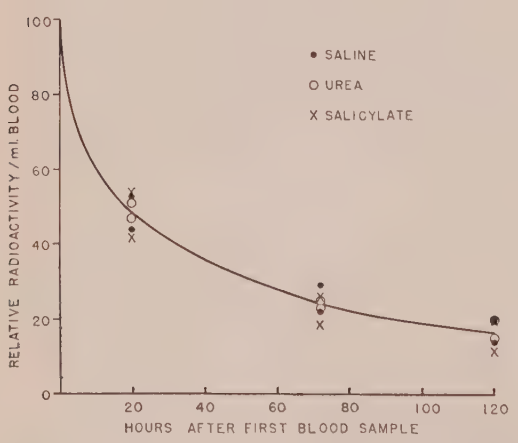

FIG. 1. Relative radioactivity of blood in rabbits after intravenous injection of saline, salicylate, or urea treated I^{131} normal rabbit gamma globulin. Initial blood sampling was made 1½ hrs after injection and this is taken as zero time.

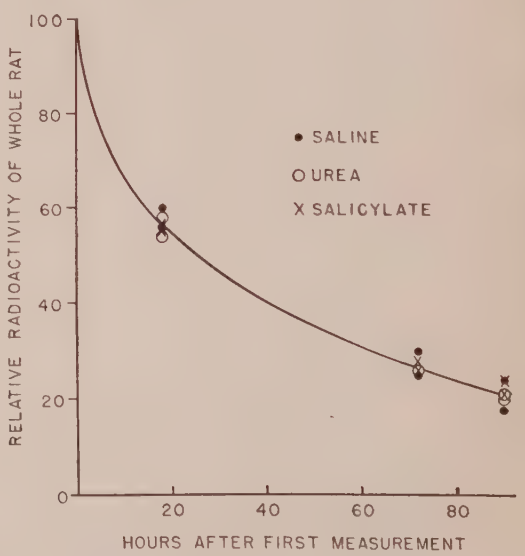

FIG. 2. Relative radioactivity of rats after intravenous injection of saline, salicylate, or urea treated I^{131} normal rabbit gamma globulin. Initial radioactivity determination was performed 1 hour after injection and this is taken as zero time.

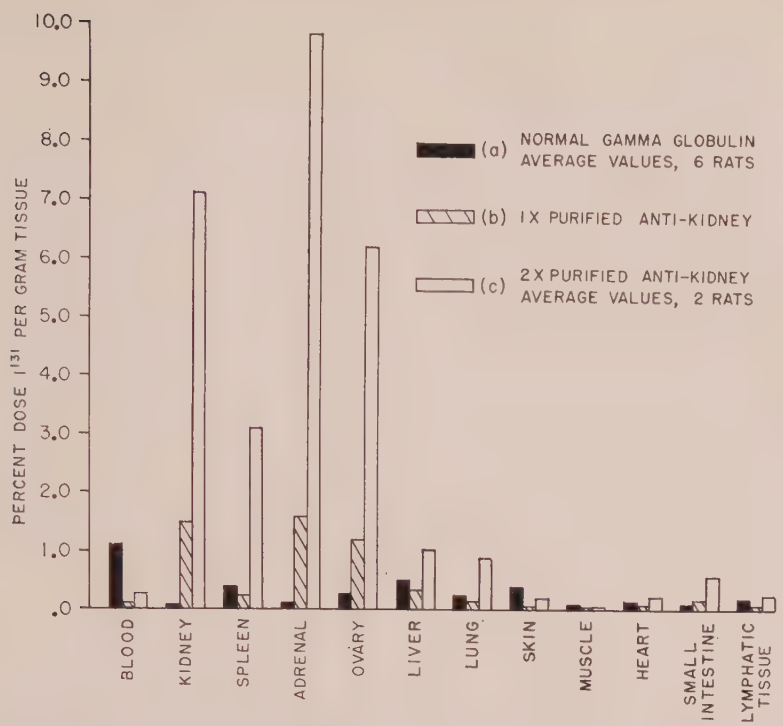

FIG. 3. Distribution of I^{131} in perfused rats following intravenous injection of labeled rabbit serum protein fractions. (a) 4 days after injection of labeled gamma globulin from a normal, non-immunized rabbit, (b) 8 days after injection of a preparation isolated by salicylate elution, before iodination, from serum of rabbits immunized against rat kidney, and (c) 5 days after injection of a fraction of the above preparation, once more purified, after iodination, using salicylate elution.

external scintillation counter, with the radioactivity of each animal, measured shortly after injection, given a value of 100. Determination of radioactivity of various tissues, made after sacrifice by saline perfusion after 90 hours, showed no significant differences between groups. Average values for all 6 animals are shown in Fig. 3. The data indicate that treatment neither with urea nor sodium salicylate has adversely affected gamma globulin as measured by the rate of elimination from the blood in rabbits or by excretion in rats.

In vivo studies with anti-rat kidney gamma globulin. Other experiments indicate that these reagents may be useful in purification of *in vivo* localizing anti-organ antibodies. In preliminary experiments, I^{131} labeled anti-rat kidney gamma globulin prepared in rabbits was absorbed on rat kidney homogenate. The labeled antibodies were then eluted with 10% sodium salicylate and, after saline dialysis, injected intravenously into rats. It was found that this antibody preparation had a several fold larger portion of I^{131} attached to antibodies that would localize in rat kidney than in the initial labeled but unpurified preparation. A similar technic was used on previously unfractionated anti-rat kidney rabbit serum. The following experiment indicates that it is possible to obtain from such an antiserum a fraction rich in rat kidney localizing antibodies. The washed residue from a homogenate of 1.7 g of rat kidney was twice extracted for 5 minutes at room temperature with 55 ml of 20% sodium salicylate. This washed, extracted residue was then incubated with stirring at 37°C for 1 hour with 17 ml of a pooled serum specimen from rabbits immunized against rat kidney. This mixture was then centrifuged, the residue washed twice with saline, then eluted for 5 minutes at room temperature by stirring with 15 ml of 10% sodium salicylate. After cen-

trifuging the supernatant was dialyzed against 15% Dextran to reduce volume, then against a pH 8 borate buffer, and the protein therein trace labeled with I^{131}. Fig. 3 shows the I^{131} distribution found in a perfused rat 8 days after intravenous injection of a portion of this labeled material. Kidney localization at 1 day was 1.82% dose per gram. The kidney localization is several fold higher than would be expected with labeled gamma globulin isolated from the same original pool of immune rabbit serum. This indicates that a higher portion of the protein isolated by this absorption-salicylate elution procedure is anti-kidney antibody than is obtained by a simple gamma globulin isolation. The high localization of I^{131} in rat adrenal and ovary is also characteristic of anti-rat kidney antibodies purified by other methods(5). A second experiment was carried out with this once purified I^{131} labeled preparation to separate labeled anti-kidney antibody from non-specific material labeled because it was present in the initial antibody preparation. Three ml of this material were incubated with a washed and salicylate eluted homogenate derived from 0.4 g of rat kidney, and the absorbed antibody again eluted with 5 ml of 10% sodium salicylate. This eluate contained 6.5% of the initial I^{131}. Salicylate was removed by dialysis. Fig. 3 also shows the average radioactivity present in 2 rats sacrificed by perfusion 5 days after intravenous injection of portions of this twice purified preparation. The I^{131} content of corresponding organs from the 2 rats showed good agreement. The individual kidney values were 7.3 and 6.9. The kidney value for a rat sacrificed at 1 day was 7.7. This second purification, carried out after I^{131} labeling, has thus resulted in a product with a 5.5 fold increase in kidney localizing tendency. It contains about 35% of the I^{131} attached to kidney localizing antibody of the initial iodinated preparation. Later duplicate experiments using either sodium salicylate or urea as eluting agents have given closely similar results.

Discussion. Urea and sodium salicylate have been widely studied because of their solubilizing action on proteins and their ability in strong solutions to modify certain proteins in a way usually described as "denaturation"(7). In weaker solutions these substances affect some proteins in a manner often termed "reversible denaturation" since the proteins are apparently regenerated after the removal of the urea or salicylate(8).

Kleinschmidt and Boyer(9), Coburn(10), and Friend(11) found that 2 M urea, 0.75 M salicylic acid, and 1 M sodium salicylate respectively inhibited the precipitation of soluble protein antigens with their specific antisera, and also dissolved preformed antigen-antibody precipitates. The effect of these agents in dissociating antigen from antibody has been little studied. Kleinschmidt and Boyer(9) reported electrophoresis showed only 5% dissociation of dissolved specific precipitates of egg albumin and its antibody. Gostev(12) apparently found urea active in rupturing the bonds between specific antibody and bacterial structure. Lepper *et al.* (13) suggested that a protective action of salicylates in rabbits against anaphylactic death might be due to a direct effect on the combination of antigen and antibody.

It is generally suggested that the effect of these reagents on protein is to rupture hydrogen bonds, and often thus cause unfolding of the protein. Studies on horse diphtheria antitoxin treated with 20% urea showed the antibody activity to be unaffected(14). Apparently also concentrations of these reagents sufficient to break antigen-antibody bonds, perhaps largely of a hydrogen bond nature, do not affect the integrity of rabbit gamma globulin molecules.

Summary. Solutions of 16-32% urea and of 10% sodium salicylate were used to separate I^{131} labeled rabbit anti-rat organ antibodies from insoluble rat organ residues acting as antigens. Following removal of these eluting agents, the antibody retained its specificity for rat organs as measured by *in vivo* and *in vitro* technics. Measured by *in vivo* technics, rabbit gamma globulin was resistant to the altering or denaturing effect of these reagents.

We thank Barbara Bailey and Jean Williamson for technical assistance.

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Use of I^{131} Labeled Oleic Acid in Study of Gastrointestinal Function.* (23095)

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Previous work using I^{131} labeled neutral fat suggested that use of a radioactive labeled fatty acid could prove valuable in studying gastrointestinal function. This report concerns use of I^{131} labeled oleic acid in the further investigation of certain physiological aspects of digestion and absorption in normal and abnormal subjects.

Procedure. Human subjects. Thirteen humans with no evidence of abnormality related to the digestive tract were used as controls. After 4 hours of fasting, each was given a gelatin capsule containing 0.5 cc of oleic acid which included 25 μc of I^{131} labeled oleic acid. They were then given 20 drops of Lugol's solution and 3 gelatin capsules containing barium sulfate powder. This was taken with small amounts of water. A fasting state was then maintained for the following 6 hour period. Two ml venous blood samples were drawn at hourly intervals at second through sixth hour following ingestion of the capsules. At the third hour fluoroscopic examination of the abdomen was made to determine gastric emptying and location of the barium. The subjects were allowed to return to their normal eating habits after the 6 hour period. All feces passed within 48 hours after the test was collected in individual containers. Blood samples were analyzed for radioiodine content and total radioiodine blood levels were determined and expressed as percentage of ingested material as described by Baylin *et al.* (1). Fecal samples were individually ana-

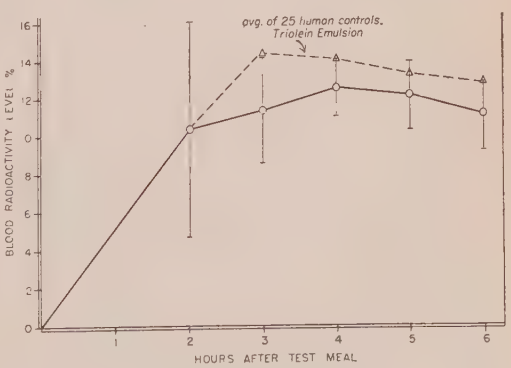

FIG. 1. Blood radioactivity levels (avg of 13 human controls with standard deviation).

* Supported by research grant of the Division of Arthritis and Metabolic Diseases, N.I.H.

TABLE I.

	Triolein emulsion				Oleic acid			
	Blood radioactivity levels (%)			Recovered in feces	Blood radioactivity levels (%)			Recovered in feces
	4 hr	5 hr	6 hr	(%)	4 hr	5 hr	6 hr	(%)
Normal (avg)	14.1	13.3	12.4	.6	12.8	12.2	11.2	.7
Ca of pancreas	4.7	4.7	3.8	49.0	10.4	11.3	11.7	10.0
Pancreatectomy	2.4	3.2	2.8	64.7	8.9	7.6	6.6	2.3
Sprue (in relapse)	3.3	3.3	4.0	32.0	3.8	7.7	7.8	15.9
Regional enteritis	3.7	3.5	3.3		6.9	5.5	5.1	48.3
Amyloidosis with marked diarrhea	5.2	5.2	4.9		1.0	.5	1.1	
Gastric resection, Hofmeister								
(1) Without evidence of malnutrition	10.6	12.0	10.9		9.4	9.3	8.9	1.3
(2) With evidence of malnutrition	3.6	3.8	3.8	72.3	11.9	10.5	9.3	.2

lyzed for radioiodine content, and the total expressed as percentage of the administered radioactivity as described previously(2). Seven patients having proven gastrointestinal disease were studied in the same manner.

Animals. Fourteen healthy dogs were used as controls. After 4 hours fasting, each control was given a gelatin capsule containing 0.5 ml of oleic acid which included 25 mc of I^{131} labeled oleic acid. Two ml venous blood samples were drawn at hourly intervals for the second thru the fifth hours following ingestion of the capsule. No attempt was made to collect the feces. Blood samples were analyzed as described above. Identical procedures were carried out on 7 dogs that were not fasted prior to the test. Two pancreatectomized dogs and 2 dogs in which pancreatitis was produced by vinylite infusion into the main pancreatic ducts(3) were tested in identical manner as the 14 control dogs.

Results. The results obtained in the 13 human controls are presented in Fig. 1. Average blood radioactivity levels and associated standard deviation are shown as a function of time after the test meal. Average fecal recovery for this group was 0.7%, with a range of 0 to 2.8%. For comparison the average curve of the human control with triolein test meal is shown.

Table I shows the results obtained in 7 patients tested with neutral fat (triolein emulsion) and with oleic acid.

The results obtained in the group of control dogs are shown in Fig. 2. The average curve of human controls with oleic acid test meal is presented for comparison. Table II shows data obtained in the 4 dogs in which pancreatic insufficiency was produced.

The 7 dogs tested under a non-fasting condition yielded radioactive blood levels which were not uniform, were unpredictable and did not conform to the reproducible curves seen in fasted animals.

The studies with normal humans and dogs have shown that characteristic and reproducible curves of blood radioactivity levels are obtained when I^{131} labeled oleic acid is given as described above. Similar results have been reported by other investigators(4). Theoretical consideration of the physiology of the G. I. tract would indicate that use of both

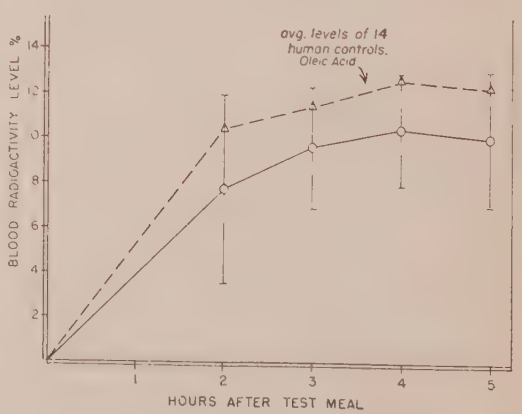

FIG. 2. Test material—½ ml oleic acid in capsule (avg levels of 13 normal dogs).

TABLE II. Blood Radioactivity Levels in Dogs.

Triolein emulsion			Oleic acid capsule		
4 hr	5 hr	6 hr	4 hr	5 hr	6 hr
(%)					
Pancreatitis—vinylite					
1.5	1.3	1.5	1.1	6.5	7.8
1.5	3.4	4.4	6.5	7.0	8.0
Pancreatectomy					
—	5.3	1.2	10.6	10.1	7.6
0	.3	0	11.3	14.7	4.5
1.6	2.0	2.2	11.7	12.0	9.0

neutral fat and fatty acid test might differentiate between abnormalities of digestion and absorption.

This assumption was tested in the 4 dogs in which pancreatic insufficiency was produced. As seen in Table II post operative neutral fat blood radioactivity levels were markedly depressed. These results attest to the pancreatic insufficiency as demonstrated previously(5). In contrast, blood radioactivity levels following oleic acid test were within the range of normal. The 2 patients with known pancreatic disease (Table I) gave results similar to those obtained in the pancreatic deficient animals. In the few patients with known small bowel disease tested, both triolein and oleic acid curves were depressed (Table I).

The results obtained in the experimental animals and the small number of patients with known gastrointestinal abnormalities suggest the probability that neutral fat and fatty acid will prove helpful in differentiating between abnormalities of digestion and absorption. Similar conclusions have been recently reported by others(4,6).

Summary and conclusion. 1. Constant and reproducible curves of blood radioactivity levels are found in normal humans and dogs after an oleic acid test. 2. Following pancreatectomy and induced pancreatitis in dogs, the curves obtained with oleic acid test were within normal range while those obtained with triolein were depressed.

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Hormonal Influences on Growth and Somatotropic Actions of Autonomous Mammotropes.* (23096)

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Estrogens stimulate secretion and proliferation of mammotropes (Mt) of the anterior pituitary gland(1-3). Mammotropic tumors (MtT) can be induced in rats by prolonged estrogen treatment; they are readily transplantable to estrogen-treated and, occasionally, to normal rats. Upon successive transplantation, the former, that is, dependent MtT, invariably gives rise to autonomous variants. Extracts of such tumors contain mammotropin (MtH; synonym: prolactin)(4). All transplanted mammotropic tumors thus far studied have had a pronounced somatotropic effect and were markedly responsive to estrogens. The present study was undertaken to investigate more precisely the role of hypo-

* This work has been supported by grant of National Institutes of Health.

TABLE I. Design of Experiments on Grafting of MtT in Variously Treated Rats.

Passage	Strain and age in days at grafting	Sex	No. of animals: Tumor-bearing	No. of animals: Controls	Treatment: Gonadex	Treatment: Adrex	Treatment: Hypex	Treatment: Stilbestrol, mg	Duration of exp. in days*
III a (Fig. 1, 5; Table II)	WF 35–40	♀	4†	2				0	140
		♀	4	3				5–6	100
		♀	3	1			×	5–6	85
		♂	3	2	×			0	166
		♂	3	2	×			5–6	86
		♂	2	1			×	5–6	85
III b (Fig. 2)	F 35–40	♀	4	2				0	78
		♀	4	3	×			0	90
		♂	4	1				0	89
		♂	4	2	×			0	89
		♂	4	2	×			6–7	69
III d	WF 44–46	♀	7	7				0	176
		♀	4	3			×	3–4	212
		♂	5	5	×			0	174
		♂	7	7	×	×		0	176
IV a (Fig. 3, 4)	F 58–75	♂	3	4	×	×‡		1.0	57
		♂	4	—	×	×‡		.1	61
		♂	5	4	×	×§		.01	61
		♂	4	—	×	×‡		.001	61
		♂	4	4	×	×‖		.0	62

* From grafting of animals until sacrifice.
† Tumor grafted in both thighs throughout passage.
‡ One animal in each group had regenerated adrenal cortex tissue.
§ Two animals in MtT-bearing group and one control had regenerated adrenal cortex tissue.
‖ One animal in MtT-bearing group had regenerated adrenal cortex tissue.

physeal, gonadal and adrenal hormones on growth of autonomous MtT (Strain 4)(3), and on its somatotropic manifestations.

Materials and methods. Rats of the Fischer strain (subline of the strain of Dr. Wilhelmina Dunning designated F) or F_1 hybrids of an inbred Wistar strain and the F strain (designated WF) were raised in our laboratories and fed Purina chow supplemented with greens. Animals differing in age by more than 5 days were evenly distributed in the experimental groups. Tumor particles suspended in saline were injected into the thigh muscles. Operations were performed and hormone treatments were started before grafting (passage IVa) or within 8 days thereafter (passages IIIa, b, and d). For chronic estrogen treatment, fused cholesterol-diethylstilbestrol (DES) pellets were implanted subcutaneously in the neck. The DES:cholesterol ratio was 1:3 in pellets used in passages IIIa, b, and d. In passage IVa, the pellets weighed 16-18 mg and contained 0.0 to 1.0 mg DES. Adrenalectomized animals were given 2.5 mg desoxycorticosterone trimethylacetate† and 2.5 mg cortisone acetate (Cortone Acetate, Sharp and Dohme) every 2 weeks. In passage IIId this treatment was discontinued after 3 months and the animals were given physiological saline as drinking water. Experimental animals were killed and autopsied in groups with their controls. For histological studies, tissues were fixed in Zenker-formol and stained with hematoxylin and eosin. The design of 4 experiments is shown in Table I. Small numbers of animals were used because of limited breeding facilities.

Results. I. *Effects of hormones on growth of MtT.* Pituitary hormones are not required for proliferation of these mammotropic tumor cells; in fact, MtT grew somewhat faster in hypophysectomized than in normal females in one passage (Fig. 1) and but slightly slower in another (passage IIId). In the latter,

† This preparation (Percorten trimethylacetate) was generously supplied by Dr. Robert Gaunt, Ciba Pharmaceutical Products, Summit, N. J.

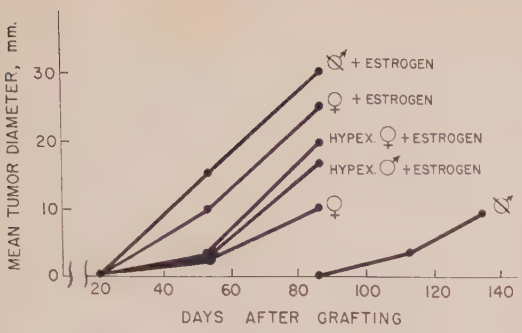

FIG. 1. Effect of diethylstilbestrol, hypophysectomy and castration on growth of autonomous MtT (passage IIIa).

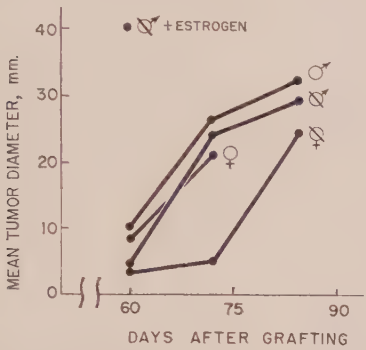

FIG. 2. Effect of gonadectomy and diethylstilbestrol on growth of autonomous MtT (passage IIIb).

mean latency of tumor growth was 122 days (range: 77-167 days) in hypophysectomized animals as compared to 102 days (range: 77-132 days) in intact untreated female hosts. In these experiments, all hypophysectomized animals were DES-treated. Earlier experiments with MtT in mice(2) suggested that without estrogen treatment, tumor growth would have been even slower than in castrated non-hypophysectomized males.

Fig. 1 shows a very long latent period and slow progression of tumor growth in castrated male animals and an extraordinary hastening of tumor growth in such rats treated with DES. Stimulation by DES was less marked in intact females. When the experiment recorded in Fig. 2 was performed, the tumor strain was less responsive to gonadal hormones. The contrast between gonadectomized and normal females was marked but there was little difference between gonadectomized and normal males. These data indicate that the effect of gonadectomy on MtT growth is due to removal of a source of estrogens.

The effect of varying quantities of DES on the growth of autonomous mammotropes is shown in Fig. 3. The rate of Mt proliferation was increased slightly by 10 μg of estrogen, moderately by 100 μg and markedly by 1 mg. In this experiment, the hosts were both adrenalectomized and gonadectomized. Adrenalectomy proved incomplete in several animals. There was, however, no difference in the rate of tumor or body growth between incompletely and completely adrenalectomized animals.

In passage IIId, MtT was grafted on castrated males and on adrenalectomized castrated males. The mean latency of tumor growth was 99 days in the former and 116 days in the latter (range: 54 to 148 days in both groups). The data suggest that adren-

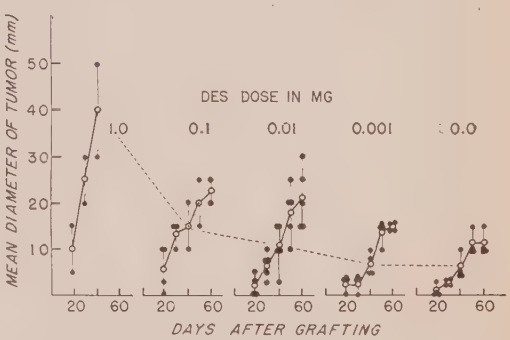

FIG. 3. Stimulation of autonomous MtT by graded doses of diethylstilbestrol (passage IVa). For key, see Fig. 4. Light dotted line connects observations on fortieth day.

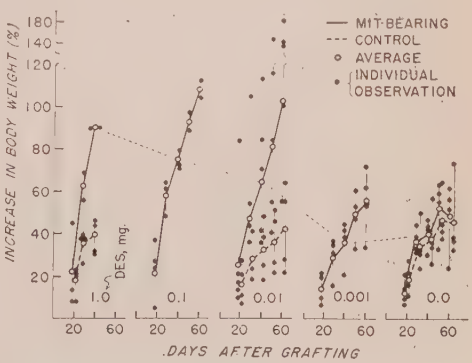

FIG. 4. Effects of different quantities of diethylstilbestrol on body weight of MtT-bearing and control rats (passage IVa). Light dotted line connects observations on fortieth day.

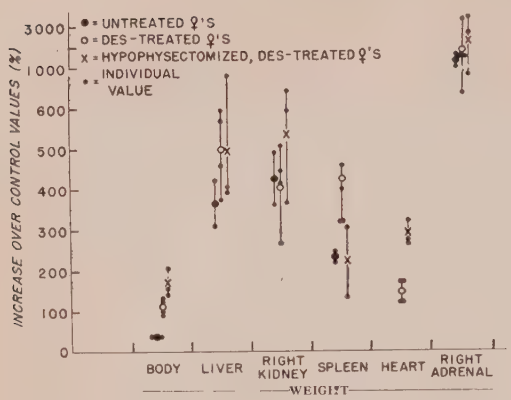

FIG. 5. Somatotropic effects of MtT. Percent increase of body and organ weights of tumor-bearing female rats over those of control animals (passage IIIa).

alectomy does not profoundly affect growth of this autonomous MtT.

II. *Somatotropic effects of MtT.* Gain in body weight (Fig. 4) paralleled that of tumor size (Fig. 3) both increasing with the quantity of estrogen administered and, presumably, with the quantity of hormone secreted by MtT. In order to indicate the somatotropic action of MtT, increases in body and organ weights of tumor-bearing female rats are expressed as percentages of the respective weights of matched controls (Fig. 5). The average weights of the mammary glands of these MtT-bearing rats (not shown in Fig. 5) were: intact females 34 g; DES-treated rats 36 g; hypophysectomized, DES-treated rats 21 g. In Table II, the somatotropic effects in variously treated males are indicated by body weight, body length, and by weights of organs expressed in percent of body weight. Considering time and tumor weight, the greatest gains occurred in hypophysectomized animals.

The data show a marked increase in overall skeletal size of most MtT-bearing animals. The liver, kidneys, spleen and heart were disproportionately enlarged, the greatest relative increase occurring in adrenal weights. These figures do not directly indicate the magnitude of somatotropic effects of MtT. Livers of MtT hosts usually had some fatty degeneration but enlargement appeared to be due mainly to increase in size of liver cells(3).

TABLE II. Effect of MtT on Body Size and Relative Organ Weights in Male Rats (Passage III a).

No. of rats	Treatment	Days after graft	Body		Tumor wt, g	% of body wt				
			Wt, g	Length, cm		Liver	Right kidney	Spleen	Heart	Right adrenal
2	DES + MtT	86	376 (365–388)	24.2 (24.2–24.2)	22.5 (19.2–25.8)	9.5 (9.3– 9.6)	.93 (.93– .93)	.54 (.53–.54)	.38 (.36–.39)	.067 (.052–.082)
3	DES	86	178 (166–187)	20.4 (19.7–21.0)	—	3.8 (3.4– 4.2)	.37 (.37– .37)	.26 (.21–.33)	.39 (.38–.41)	.017 (.014–.019)
3	Castrate + MtT	166	300 (289–305)	24.0 (23.5–24.5)	16.1 (13.7–21.7)	9.6 (8.5–11.1)	1.26 (.80–1.56)	.36 (.27–.48)	.45 (.33–.62)	.110 (.068–.166)
2	Castrate	166	308 (304–312)	23.0 (22.5–23.5)	—	3.2 (3.2– 3.3)	.32 (.32– .33)	.25 (.24–.26)	.29 (.29–.29)	.007 (.007–.007)
3	Hypex + DES + MtT	85	153 (143–163)	19.1 (18.4–19.7)	5.3 (1.6–9.0)	7.1 (6.1– 8.0)	.69 (.61– .77)	.38 (.27–.48)	.63 (.59–.66)	.054 (.032–.077)
1	Hypex + DES	85	73	15.5	—	3.4	.29	.22	.42	.006

The kidneys often underwent a marked tubular degeneration. The numbers in Fig. 5 do not indicate as marked a relative increase in body weights in intact as in DES-treated female tumor hosts. This difference may, in part, be accounted for by the greater length of time required for growth of MtT in normal rats and corresponding longer growth periods in their controls. In this experiment (passage IIIa) the intact female rats were killed 140 days after grafting when the tumor weight averaged 17.4 g; the DES-treated females were killed after 100 days when the tumor weight averaged 23.9 g; the hypophysectomized DES-treated females were killed after 85 days when the tumors weighed 4.6 g. Weights of the mammary gland represent the sum of secretions and increase in tissue mass. A detailed analysis of the character of mammary gland changes will be published.

The enormous hypertrophy of the adrenals remains to be explained. Increase in size was accompanied by extensive fatty degeneration of the cortex, often with hemorrhage and necrosis. The enlargement did not appear to be due primarily to adrenotropic effect by the tumor; the thymus of rats with medium-sized tumors was often normal. The thymus is known to undergo atrophy in animals bearing large tumors of all kinds. Grafted adrenotropes (in mice) cause atrophy of the thymus while the tumor is minute(2).

In passage IIIb, of 7 animals bearing tumors measuring 15-20 mm in diameter, the thymus was normal in 4, (average right adrenal weight 57 mg; in 9 controls 14-29 mg, mean, 19 mg), moderately atrophic in 1 (right adrenal weight, 108 mg) and atrophic in 2 (right adrenal weights, 210 and 215 mg). Of 7 animals with tumors 25-30 mm in diameter, the thymus was moderately atrophic in 1 (right adrenal weight, 130 mg) and atrophic in 6 (average right adrenal weight, 203 mg). Of 2 animals bearing tumors measuring 60 mm in diameter, the thymus of 1 was moderately atrophic (right adrenal weight, 123 mg) and completely atrophic in the other (right adrenal weight, 295 mg). There was no clear-cut relationship between size of the thymus and treatment of the host.

Discussion. The present studies indicate that hormones of MtT greatly stimulate growth of the body and viscera, and that the fully autonomous Mt retains a marked responsiveness to estrogen and is not dependent on pituitary hormones for proliferation and function.

Mammotropic and somatotropic effects occurred in parallel and in rough proportion to tumor size. It is possible(2,3) that MtH has intrinsic somatotropic action in rats and mice as has been previously reported in birds(5). Similar overlaps in function are well known; *e.g.*, in oxytoxic and vasopressor principles of the posterior pituitary and among adrenocorticoids. It is also possible that MtT (Mt) produces 2 distinct hormones but the parallel rise of somatotropic effect with mammotropic effects following administration of estrogen is better explained by postulating secretion of one hormone with these two potencies. Contopoulos and Simpson‡ described a body growth-promoting factor in the blood of pregnant rats. It seems likely that this effect was, in part at least, due to MtH. An adrenal growth factor in pituitary extracts distinct from AtH (ACTH) has also been suggested (6).

The autonomous mammotropic tumor studied by us was markedly reactive to the agents which initiated it. Responsiveness of malignant cells may yield clues to the factors involved in their genesis. Thus, autonomy is not synonymous with full release from responsiveness to conditioning factors, and study of responsiveness may help in determining the conditions leading to development of neoplasms as well as the factors controlling them.

Summary and conclusions. 1. The role of gonadal, adrenal, and hypophyseal hormones on growth and somatotropic actions of a transplantable, functional mammotropic pituitary tumor (MtT) was studied in rats. 2. Although this tumor is autonomous (in that it grows in normal rats of both sexes) it is highly responsive to estrogen, the rate of tumor growth and body growth of the host being roughly proportional to the quantity of estro-

‡ Reported in *Fed. Proc.*, 1956, v15, 39.

gen administered. Hormones of mammotropes stimulate body growth and growth of many organs. Somatotropic and mammotropic effects are marked in hypophysectomized rats bearing mammotropic tumors; thus they are independent of other pituitary factors.

Mrs. Lee Zompetti skillfully assisted in this work.

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Binding of Novobiocin with Plasma Proteins. (23097)

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The antibiotic activity of novobiocin *in vitro* is inhibited by the presence of serum (1).* In *in vivo* experiments novobiocin is still present in the blood stream 24 hours after an oral dose(2).* These findings together with evidence from dialysis* have suggested that novobiocin must bind to plasma proteins.

The purpose of this work was to determine the nature and extent of this binding. Fractionation studies with plasma from a dosed dog showed that novobiocin is extensively bound to plasma albumin, and *in vitro* experiments with crystalline bovine albumin showed that the binding is a reversible equilibrium process.

Plasma fractionation. An adult female Beagle dog was given by mouth 100 mg of novobiocin (CATHOMYCIN sodium, Merck) per kg of body weight. After 3 hours blood was withdrawn from the jugular vein, mixed with .15 volume of sodium citrate-citric acid-dextrose (ACD) solution(3), and centrifuged. The clear plasma, amounting to 38 ml, was fractionated by Cohn's Method 10(3). A second dog, not dosed, supplied a like amount of plasma as a control. The plasma fractions were assayed for novobiocin by the microbiological procedure of Frost and Valiant(4).

* To be published.

TABLE I. Novobiocin Concentrations in Plasma Fractions.

Fraction	Novobiocin content as γ/ml of original plasma: Dosed dog	Control dog
Original plasma	164	0
Fractions I, II, & III	<2	0
" IV & V	62	0
" VI*	++++	++++
" IV	4.2	0
" V	35	0

* Fraction VI contained Zn^{++} which interfered with assay.

The results are shown in Table I. The first precipitate, consisting of fractions I, II, and III, did not contain appreciable amounts of novobiocin. Fractions IV and V, precipitated together by the addition of Zn^{++} reagent, contained 38% of total plasma novobiocin. The excess Zn^{++} reagent present with fraction VI interfered with microbiological assay of this fraction. On separation of fractions IV and V it was found that almost all of the novobiocin was bound to fraction V, which consists of the plasma albumin. Quantitative recovery cannot be expected in this fractionation when binding is reversible, first because not all of the novobiocin originally present in the plasma is bound, and second because of additional dissociation brought about by volume increases on reagent addition and washing of precipitates.

TABLE II. Novobiocin Concentrations in Dialysates.

Dialysate of		Novobiocin conc., γ/ml Microbiological	Colorimetric
.5% albumin	a	62.5	59
	b	46	58
.05% "	a	92	93
	b	100	88
.005% "	a	86	
	b	120	

Binding with bovine albumin. One mg of novobiocin and 500 mg of crystalline bovine albumin were dissolved in 10 ml of M/15 pH 7.4 phosphate buffer and dialyzed in a cellophane sack against one liter of the same buffer for 64 hours in the cold. Microbiological assays at the end of this time showed that concentration of novobiocin inside the sack was 90 γ/ml and that in the dialysate was below the threshold of the assay procedure. Thus novobiocin will bind to bovine plasma albumin.

Equilibrium concentrations. In duplicate experiments 10 ml volumes of M/15 pH 7.4 phosphate buffer containing 100 γ of novobiocin per ml and either 0.5%, 0.05%, or 0.005% bovine albumin were dialyzed at 37°C for 64 hours in cellophane sacks against 30 ml volumes of buffer containing 100 γ of novobiocin per ml. At the end of this time the solutions outside the sacks were assayed by microbiological assay and by the colorimetric procedure of Boxer and Shonk(5). Volume changes were negligible. The results are presented in Table II.

Ignoring any effect of the Donnan equilibrium, which would be minimized by the buffer salts present, we may assume that concentration of novobiocin outside the sack is identical with concentration of unbound novobiocin inside the sack. For the sacks containing 0.5% albumin amount of bound novobiocin can be calculated as follows:

Total novobiocin introduced, $100 \times 40 = 4000\ \gamma$
" free novobiocin, $58.5 \times 40 = 2340$
Novobiocin bound to 50 mg of albumin $= 1660$

Similarly, in the sacks containing 0.05% albumin:

Total novobiocin introduced, $100 \times 40 = 4000\ \gamma$
" free novobiocin, $90.5 \times 40 = 3620$
Novobiocin bound to 5 mg of albumin $= 380$

The amount of novobiocin bound per milligram of albumin was greater when concentration of free novobiocin was higher, an indication that the binding is an equilibrium process.

This was further investigated by placing the sacks containing the 0.5% albumin solution in fresh portions of buffer, containing no novobiocin this time, dialyzing for another 24 hours, and again analyzing the dialysates by the colorimetric procedure. They contained 24 γ, 25 γ of novobiocin per ml.

For the second dialysis the calculation is as follows:

Total novobiocin in system, $1660 + 10 \times 58.5 = 2245\ \gamma$
" free novobiocin, $24.5 \times 40 = 980$
Novobiocin bound to 50 mg of albumin $= 1265$

Thus the second dialysis produced a decrease of 395 γ in the bound novobiocin, and therefore the binding is reversible.

Summary. 1. Fraction studies with plasma from a dog treated with novobiocin showed that novobiocin is extensively bound to plasma albumin. 2. *In vitro* experiments with crystalline bovine albumin showed that the binding is a reversible process.

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A Method for Evaluation of Milk "Let-Down" in Lactating Rat.* (23098)

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It has been generally accepted(1-5) that oxytocin is released from the posterior pituitary gland in response to neural impulses generated by stimulation of nursing or milking. Upon reaching the mammary gland oxytocin causes contraction of myoepithelial elements (6-8) surrounding mammary alveoli and small ducts thus forcing milk into larger ducts where it is available for withdrawal. The phenomenon of milk "let-down" appears to be one of the more important factors determining amount of milk obtained from milking or nursing.

In the present study data have been obtained in an attempt to evaluate "let-down" in the rat as a result of nursing stimuli in terms of amount of milk obtained under standard conditions by litters of lactating rats during the period of maximum lactation.

Materials and methods. Forty albino rats each with its first litter and weighing 180-260 g were housed in individual cages and fed Purina Lab Chow and water *ad libitum*. Each litter was reduced to 6 young shortly after birth and when 14 days old was isolated from its mother for 10 hours. The young were then replaced and allowed to suck for 30 minutes. Length of time before each litter began sucking was recorded. Each litter was then removed from the nest, weighed, killed by decapitation and stomach contents removed and weighed. Each mother's weight also was recorded.

Results. It was observed previously(9) that after 10 hours isolation stomachs of litters contained no milk. In each case upon placing the litter back with their mother, she was observed to gather her young and to commence nursing in 1-3 minutes. The young appeared to be satiated within 30 minutes and very frequently disengaged the nipple or else fell asleep while still attached to the mother. Amount of milk obtained by litters of lactating rats in 30 minutes varied from 2.5-10.5 g/litter. There was no appreciable difference in milk volume between any 2 young of a litter. Weight of milk expressed as percent litter body weight shows more uniformity with a mean of 3.8 $\pm$ .19, a median value of 3.7 and normal distribution (Table I).

Discussion. Previous work in this laboratory(9) indicated that weight of milk expressed as percent litter body weight after 20 minutes nursing gave a mean of 3.7 whereupon experimental results following ergotamine treatment were statistically evaluated. The expanded results of this study are almost identical. Comparable results also have been obtained from basically the same procedure by Mayer(10) and by others from calculating litter weight gain during timed nursing periods(11,12).

One might justifiably point out that heavier lactating rats would have more mammary tissue, produce more milk and thus make more available to the young per unit time. However, there was no apparent direct correlation when milk yield per timed nursing was expressed as percent mother body weight (Table I). The observation that 14-day-old litters are satiated by 30 minutes sucking does not mean all milk is withdrawn from the nursed glands for unpublished work indicates more milk is available than is normally withdrawn at a nursing.

It has been demonstrated(13) that a litter of 6 young will suck all 12 nipples of a lactating rat during lactation thus maintaining all mammary glands in the state of active secretion. Variance in data obtained in this study is therefore interpreted to reflect variance in amount of oxytocin released and/or variance in requirements of the mammary gland for the hormone. Other factors, of course, such as nipple diameter and intensity of nursing stimuli might influence amount

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† Postdoctoral Fellow of the N.I.H.

TABLE I. Milk Removal by Litters of Nursing Rats at 14th Day of Lactation.

No. of rats	Avg wt of litters (g)	Avg wt of milk (g)	Avg % Wt of milk / Wt of litter	Avg wt of mothers (g)	Avg % Wt of milk / Wt of mother
4	139.6	2.8	2.0 (1.3-2.2)	204.5	1.4 (1.2-1.6)
8	135.4	3.7	2.7 (2.3-3.2)	214.5	1.8 (1.4-2.2)
15	150.9	5.6	3.7 (3.3-4.2)	216.4	2.7 (2.3-3.4)
7	144.4	6.7	4.6 (4.3-5.2)	198.4	3.4 (2.8-4.0)
5	133.9	7.5	5.6 (5.3-6.2)	225.3	3.4 (3.0-3.7)
1	156.0	10.5	6.7	230.0	4.6
Mean	144.0	5.5	3.8 ± .19	213.1	2.6 ± .19

of milk obtained but these factors are difficult to evaluate.

The results under the conditions of this experiment are believed to evaluate to some extent milk "let-down" resulting from nursing stimuli and thus serve as a basis for future work concerned with experimental alteration of milk "let-down" in mature primiparous lactating rats.

Summary. A method for evaluation of milk "let-down" in mature lactating rats on 14th day postpartum is described. Amount of milk obtained by litters of 6 young after 30 minutes nursing expressed as percent litter body weight is used as criterion of response. Results obtained follow a normal distribution pattern with a mean value of 3.8 ± .19. The results of this study may serve as a basis for evaluating data from experimental alteration of milk "let-down" in lactating rats.

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Coagulation Defect in Horse Plasma.* (23099)

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Discrepancies in the explanation of the coagulation defect of horse plasma exist. Antihemophilic factor (AHF) is said to be present (1) or absent(2). We have recently reported an apparent lack of the Christmas factor (PTC)(3). Four out of 5 different horse plasma samples showed delayed thrombin formation, which could be restored to normal by addition of normal human serum. However, investigations with human hemophilic patients have revealed that other defects in the plasma thromboplastin system than a deficiency in PTC are restored by addition of normal serum(4). This observation prompted a re-investigation of the defect in horse plasma.

Materials and methods. The prothrombin time (Quick) was determined according to (5). The prothrombin-proconvertin complex was estimated by the Owrens method(6,7), using $BaSO_4$ absorbed plasma(8). The thrombin generation test was performed as described before(4). Barium sulfate absorbed serum, heated reabsorbed serum and platelet suspensions were prepared as described(9).

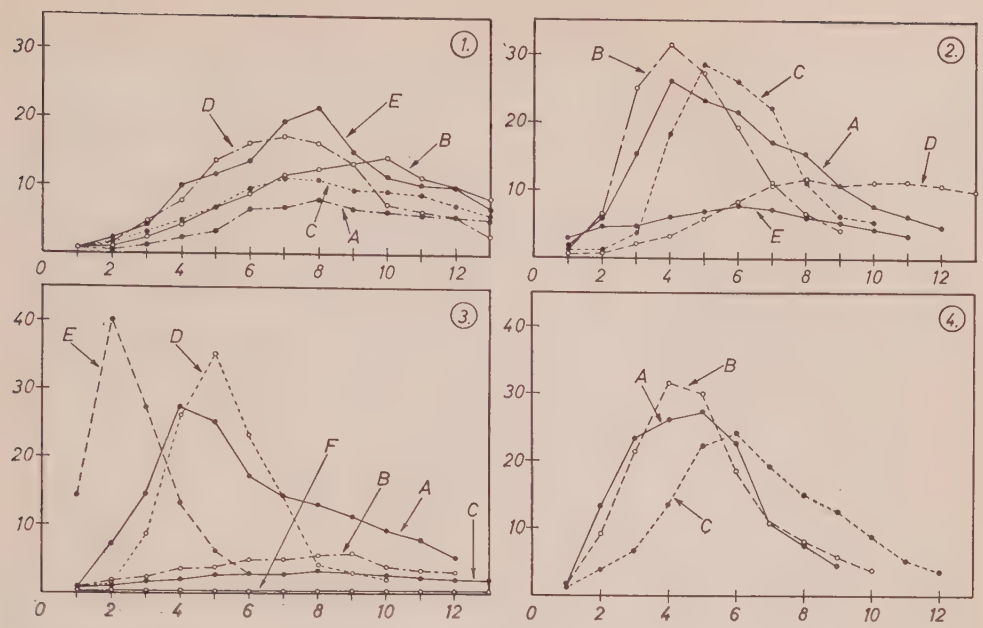
Results. The following results were obtained with blood from one horse. They are representative for the deficiency. Prothrombin time: 20 sec. (Normal human control: 19 sec.) Prothrombin-proconvertin (Owren): 90%. Platelet count: 136000 per mm^3 plasma. The thrombin generation was delayed and the thrombin concentration never reached high values (Fig. 1 A). Recalcification time (as estimated during thrombin generation test) was 3 min. 50 sec. Addition of 0.2 ml absorbed bovine plasma to 1 ml of horse plasma had only a slight effect on thrombin generation (Fig. 1B). Recalcification time was now 3 min. 30 sec. Addition of 0.2 ml platelet poor, frozen plasma from another deficient horse had no effect on thrombin formation (Fig. 1 C). After addition of 0.2 ml of its own frozen, platelet rich plasma, thrombin generation became almost normal (Fig. 1 D). Similar result was obtained also after addition of normal platelet containing human plasma (Fig. 1 E).

Fig. 2 shows thrombin generation after addition of 0.2 ml normal human serum (curve A), 0.2 ml twice absorbed normal human serum (curve B) and 0.2 ml twice absorbed human serum, previously heated for 30 min. at 56°C (curve C). In all these mixtures thrombin generation became normal. Recalcification time was 1 min. 45 sec., 2 min. and 2 min. 50 sec. respectively. Addition of 0.2 ml fresh plasma (curve D) or serum (curve E) from a patient with the Hageman trait had no effect on thrombin generation (Fig. 2).

Fig. 3 shows results obtained after storage of the horse plasma at –20°C for one night. Thrombin generation became completely normal after freezing the plasma with its normal content of platelets (curve A), whereas there was no improvement after freezing of the platelet poor plasma (15000 platelets per mm^3) (curve B). Curve C demonstrates thrombin generation in fresh horse plasma after addition of 0.2 ml of its own thrombin free serum. There was no improvement. Curves D and E show also thrombin generation in plasma from a patient deficient in PTC after addition of horse plasma (D) and horse serum (E) (0.2 ml). Thrombin generation in the PTC deficient plasma became completely normal. Thrombin generation in the Christmas plasma with no additives is demonstrated in Fig. 3 curve F.

Fig. 4 demonstrates effects of various platelet suspensions: Curve A was obtained after addition of 0.4 ml fresh human platelet

* This investigation was aided by grants from "Kong Christian den Tiendes Fond". It forms part of investigation on blood coagulation for which Dr. Tage Astrup of the Biological Institute, Carlsberg Foundation, receives support from Josiah Macy Foundation, N. Y. The careful technical assistance of Miss Erna Zoffmann is greatly appreciated.



FIGS. 1-4. Thrombin generation in horse plasma. *Abscissa:* reaction time in min.; *Ordinate:* reciprocal clotting time of fibrinogen solutions expressed as 600/t (t in sec).

suspension (245000 platelets per mm^3). Curve B shows addition of 0.4 ml frozen human platelet suspension (272000 platelets per mm^3). Curve C shows addition of 0.4 ml of a fresh suspension of platelets from the horse plasma investigated here (156000 platelets per mm^3). Thrombin generation became normal in all these experiments.

Discussion. Samples of hemophilioid diseases, which yield normal thrombin generation after addition of normal serum, comprise more than one group of defects in the plasma thromboplastin system(4). One of these groups is deficient in PTC. The results described here show that the defect in horse plasma, though corrected by addition of serum, is different from PTC deficiency. Thrombin generation in horse plasma was improved after addition of normal human plasma. It was also improved by addition of a component in human serum, which is not adsorbed by $BaSO_4$ and resists heating at 56°C for 30 min. This factor was absent from the horse serum and from plasma or serum from a patient with the Hageman trait. These results indicate that the coagulation defect in this horse is caused by lack of a factor probably identical with the human Hageman factor. Thrombin generation in horse plasma is also improved by addition of a washed suspension of human platelets, fresh as well as frozen. It is even improved by addition of a fresh suspension of washed platelets from its own plasma. Similarly thrombin generation became normal after freezing of the platelet rich plasma with no further additives. Freezing of the platelet poor plasma had no significant effect on thrombin generation. Apparently absence of the Hageman factor can be compensated by the frozen or normal washed platelets. This phenomenon has recently been observed also in human plasma(10). The finding that the samples of horse plasma and horse serum here investigated were able to make normal thrombin generation in plasma from a supposed PTC deficient patient does not accord with our earlier observation(3). However, further investigations have shown this patient to be deficient in a factor which is present in reabsorbed normal human serum, and which therefore differs from PTC.

The sample of horse plasma previously used(3) did not yield a completely normal thrombin generation after freezing, as was found in the present sample. This observa-

tion and the finding mentioned before of a horse with completely normal thrombin generation, together with the reports of other authors indicate that horse, like man, presents various types of hemophilioid diseases. P. Barkhan (personal communication) has also observed different defects in the thromboplastin system of the various samples of horse plasma.

Summary. 1. The delayed thrombin generation in samples of horse plasma was made normal by 1) addition of normal human plasma, or 2) addition of reabsorbed heated human serum, or 3) addition of washed human or horse platelets, or 4) freezing of the horse plasma with its normal content of platelets. 2. These results indicate that the clotting defect of the horse plasma investigated is caused by lack of a factor similar to the Hageman factor. 3. Blood platelets, after washing or freezing, apparently substitute for the effect of the lacking plasma factor. 4. The coagulation defects in horse plasma apparently reflect various types of hemophilioid deficiencies.

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Comparison of Duration of Action of Progesterone and 17-α-Hydroxyprogesterone-17-n-Caproate. (23100)

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17-α-Hydroxyprogesterone(1), a progestationally ineffectual compound(2), has been esterified to yield 17-α-hydroxyprogesterone-17-n-caproate (HPC), a potent progestational substance(3). Junkmann(3) also demonstrated that when HPC was administered to rabbits in an ethyl lactate-castor oil vehicle, the progestational action was more prolonged than when progesterone was given in the same vehicle.

The present study was designed to verify Junkmann's findings and to determine the duration of action of HPC in benzyl benzoate-sesame oil, an oily vehicle more desirable for human therapy. Limited success has been reported previously by Plotz(4) in prolonging the activity of progesterone by vehicular alteration.

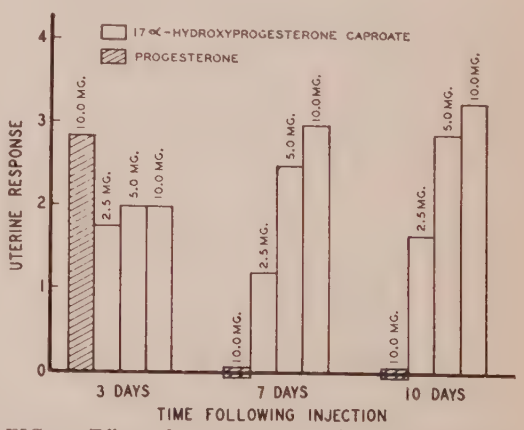

FIG. 1. Effect of progesterone and 17-α-hydroxyprogesterone caproate in ethyl lactate-castor oil on progestational changes in rabbit uterus (single subcutaneous inj).

Materials and methods. Immature virgin female Flemish rabbits were primed with daily subcutaneous injections of 5 μg of estradiol in 0.5 cc of sesame oil for 6 days. Treatment was initiated on the morning of the seventh

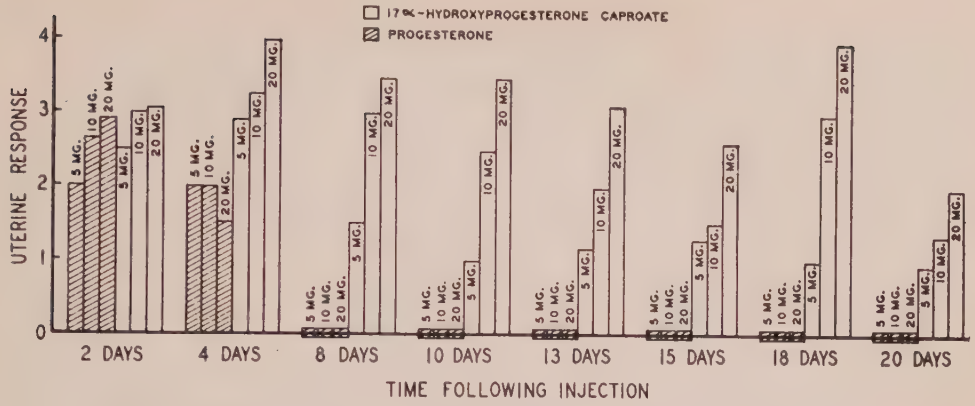

FIG. 2. Effect of progesterone and 17-α-hydroxyprogesterone caproate in benzyl benzoate-sesame oil on progestational changes in rabbit uterus (single subcutaneous inj).

day. In one study 10 mg of progesterone, or 2.5, 5 or 10 mg of HPC in ethyl lactate-castor oil,* or the vehicle alone was given in 1 cc as a single subcutaneous injection. Estradiol administration was continued daily at 0.5 μg for the duration of the study. In a second study an identical procedure was followed to evaluate benzyl benzoate-sesame oil† as a vehicle, but in this study 5, 10 or 20 mg of progesterone and similar doses of HPC‡ were administered. Groups consisting of 4 rabbits were sacrificed at intervals ranging from 2 to 20 days following the injection of the steroids, and vehicle-treated control animals received similar necropsy treatment. A uterine segment from each rabbit was fixed in Bouin's fluid and examined microscopically after hematoxylin-eosin staining. Simple estrogenic proliferation of the uterine mucosa was assigned an arbitrary value of 0, while the degree of progestational hyperplasia of the endometrium was estimated on a +1 to +4 basis(5).

Results. The progestational effect of a single progesterone injection was more pronounced at 3 days following administration than was the effect of HPC, when ethyl lactate-castor oil was used as the vehicle. Although progestational stimulation could not be detected 7 days after progesterone administration, the uterine response to HPC was greater at 7 days than at 3 days and was even more marked at 10 days. These data are in agreement with those of Junkmann(3), who reported similar activity after a single injection of HPC in the same oily vehicle, with maintenance of this effect beyond 13 days (Fig. 1). Only an estrogenic hypertrophy was seen in the control animals receiving vehicle alone.

When a single injection of progesterone was given in benzyl benzoate-sesame oil the uterine response was maximal at 2 days but showed signs of waning at 4 days, whereas the effect of HPC in this vehicle reached a peak at 4 days. Eight days following administration of progesterone in benzyl benzoate-sesame oil no progestational response was noted, while the response to HPC remained high. Thereafter, the progestational effect of HPC declined but was still apparent at 20 days (Fig. 2). Again no progestational response was seen in the vehicle-tested controls.

In comparing the data from Fig. 2 with those in Fig. 1 and those reported by Junkmann(3) it is apparent that an earlier peak response to HPC is obtained with benzyl benzoate-sesame oil than with ethyl lactate-castor oil. On the other hand, duration of activity of HPC in both vehicles is approximately the same.

* Composition in final solution: ethyl lactate 458 mg/cc; castor oil 424 mg/cc.

† 30% benzyl benzoate v/v; 70% sesame oil v/v; 0.5% chlorobutanol as a preservative.

‡ As Delalutin (Squibb), containing 125 mg of HPC per cc of 30% benzyl benzoate, 70% sesame oil and 0.5% chlorobutanol.

Similar studies of shorter duration were also carried out to evaluate the effectiveness of 17-α-hydroxyprogesterone as a progestational agent. A +2 progestational effect was noted with 50 mg of this compound, whereas no effect was demonstrated at 25 mg. This indicates that 17-α-hydroxyprogesterone is approximately 1/100 as active as progesterone *per se*.

Summary. 1. 17-α-hydroxyprogesterone-17-n-caproate (HPC) is an active progestational compound possessing prolonged activity, in confirmation of Junkmann(3). 2. Modification of the oily vehicle has resulted in an earlier peak response to HPC in virgin female rabbits, without affecting duration of action. 3. It has been verified that 17-α-hydroxyprogesterone is an extremely weak progestational agent.

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